



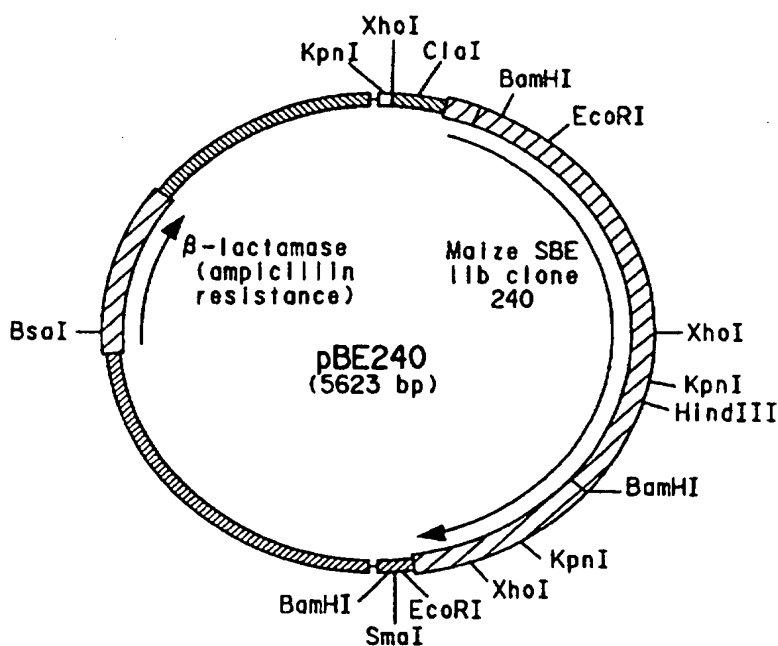
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(54) Title: NOVEL STARCHES VIA MODIFICATION OF EXPRESSION OF STARCH BIOSYNTHETIC ENZYME GENES

(57) Abstract

The instant invention discloses utilization of a cDNA clone to construct sense and antisense genes for inhibition of starch branching enzyme enzymatic activity in corn. More specifically, this invention concerns a method of controlling the starch fine structure of starch derived from the grain of corn comprising: (1) preparing a chimeric gene comprising a nucleic acid fragment encoding a starch branching enzyme structural gene or a fragment thereof, operably linked in either sense or antisense orientation on the upstream side to a nucleic acid fragment encoding a promoter that directs gene expression in corn endosperm tissue, and operably linked on the downstream side to a nucleic acid fragment encoding a suitable regulatory sequence for transcriptional termination, and (2) transforming corn with said chimeric gene, wherein expression of said chimeric gene results in alteration of the fine structure of starch derived from the grain of said transformed corn compared to the fine structure of starch derived from corn not possessing said chimeric gene.



TITLE
NOVEL STARCHES VIA MODIFICATION OF EXPRESSION OF STARCH
BIOSYNTHETIC ENZYME GENES
BACKGROUND OF THE INVENTION

5 Characteristics and Commercial Utility of Starch

Starch is a mixture of two polysaccharides, amylose and amylopectin. Amylose is an unbranched chain of up to several thousand α -D-glucopyranose units linked by α -1,4 glycosidic bonds. Amylopectin is a highly branched molecule made up of up to 50,000 α -D-glucopyranose residues linked by α -1,4 and α -1,6 glycosidic bonds. Approximately
10 5% of the glycosidic linkages in amylopectin are α -1,6 bonds, which leads to the branched structure of the polymer.

Amylose and amylopectin molecules are organized into granules that are stored in plastids. The starch granules produced by most plants are 15-30% amylose and 70-85% amylopectin. The ratio of amylose to amylopectin and the degree of branching of
15 amylopectin affects the physical and functional properties of the starch. Functional properties, such as viscosity and stability of a gelatinized starch determine the usefulness and hence the value of starches in food and industrial applications. Where a specific functional property is needed, starches obtained from various crops such as corn, rice, or potatoes may meet the functionality requirements. If a starch does not meet a required
20 functional property, if for example it must have stable viscosity under high temperatures and acidic conditions, the functionality can sometimes be achieved by chemically modifying the starch. Various types and degrees of chemical modification are used in the starch industry, and the labeling and use of chemically modified starches must meet government regulations.

25 Within the starch bearing organs of plants, the proportion of amylose to amylopectin and the degree of branching of amylopectin are under genetic control. For example, plants homozygous recessive for the *waxy* (*wx*) gene lack a granule-bound starch synthase enzyme and produce nearly 100% amylopectin. Plants homozygous recessive for the *amylose extender* (*ae*) gene can produce starch granules that are up to
30 90% amylose (see U. S. Pat. No. 5,300,145). The *dull* gene has been shown to influence the levels of activity of a starch synthase and a starch branching enzyme.

Most cereal crops are handled as commodities, and many of the industrial and animal feed requirements for these crops can be met by common varieties which are widely grown and produced in volume. However, there exists at present a growing
35 market for crops with special end-use properties which are not met by grain of standard composition. Most commonly, specialty corn is differentiated from "normal" corn, also known as field corn, by altered endosperm properties, such as an overall change in the ratio of amylose to amylopectin as in waxy or high amylose corn, an increased

The use of chemically modified starches in the United States is regulated by the Food and Drug Administration (FDA). "Food starch-modified" starches may be used in food but must meet specified treatment limits, and "industrial starch-modified" starches may be used in items such as containers that come in contact with food and must also meet specified treatment requirements; Code of Federal Regulations, Title 21, Chapter 1, Part 172, Food Additives Permitted in Food for Human Consumption, Section 172.892, Food Starch-Modified, U. S. Government Printing Office, Washington, D. C. 1981; (a) Part 178, Indirect Food Additives, Sect. 178.3520, Industrial Starch-Modified. These regulations limit the degree of chemical modification by defining the maximum amount of chemical reagent that can be used in the modification steps. The levels of by-products in starch resulting from the modification process are also regulated. For example, propylene chlorohydrin residues in hydroxypropyl starch are of special concern; Tuschhoff, J. V., (1986) Hydroxypropylated Starches, In Modified Starches: Properties and Uses, O. B. Wurzburg, ed., CRC Press, Boca Raton, FL, pp. 55-57.

15 Alteration of Starch Fine Structure Through Molecular Genetic Manipulation of Starch-Bearing Plants

Differences in the degree of starch branching or polymerization are known to result in a change in the physiochemical properties of starch. It has been suggested that starches, tailor-made for specific applications, may be generated by alteration of the branch chain distribution of the amylopectin molecule, the relative proportion of amylose to amylopectin or the degree of polymerization of amylose. However, achieving phenotypic alteration of starch composition has been problematic; while key enzymes in starch biosynthesis have been identified, their exact roles remain uncertain. Thus, correlation of activities of particular enzymes with particular molecular characteristics of starch structure and, in turn, with starch function in food and industrial products has been difficult. Although desirable functional properties that an ideal starch might need can be envisioned, there is only a vague understanding of what the molecular structure of the starch should be to achieve this and little understanding of how particular starch biosynthetic enzymes specifically affect those parameters. For example, the role of individual enzymes in determining the branching patterns and length of branches is as yet unclear and is compounded by the lack of understanding of how branching enzymes and starch synthases interact.

WO 94/09144 discusses the generation of plants with improved ability to synthesize starch at elevated temperatures. This publication proposes that the limiting factor in grain filling at elevated temperature is the lability of certain starch biosynthetic enzymes, particularly starch synthase (SS) and starch branching enzyme (SBE). The

Alteration of starch fine structure in corn is complicated by the fact that three SBE isoforms have been identified. In corn endosperm, the three isoforms that demonstrate starch branching enzyme activity are SBEI, SBEIIa and SBEIIb. In the *amylose extender* (*ae*) mutant, SBEIIb activity has been found to be deficient while in the *dull* (*du*) mutant, decreased levels of SBEIIa are observed (Boyer, C. D. and Preiss, J. (1981) *Plant Physiol.* 67:1141-1145). Studies of the catalytic properties of the corn starch branching enzymes indicate that the isoforms differ in substrate preference and in the length of glucan chain that is transferred. SBEI activity is higher when amylose serves as the substrate, and longer chains are preferentially transferred. The SBEII isoforms display higher activity with more highly branched substrates such as amylopectin. These enzymes preferentially transfer shorter glucan chains (Guan et al. (1993) *Plant Physiol.* 102:1269-1273; Takeda et al. (1993) *Carbohydrate Res.* 240:253-263).

A corn SBEI cDNA has been cloned and sequenced (Baba et al. (1991) *Biochem. Biophys. Res. Commun.* 181:87-94; Fisher et al. (1995) *Plant Physiol.* 108:1313-1314). In addition, a corn SBEII cDNA clone has been isolated and the nucleotide sequence of the clone has been published (Fisher et al. (1993) *Plant Physiol.* 102:1045-1046). This cDNA clone maps to the *ae* locus, confirming that this locus encodes the structural gene for corn SBEIIb (Stinard et al. (1993) *Plant Cell* 5:1553-1566).

Starch isolated from the *ae* mutant is known to differ in structure from that isolated from dent corn (Baba et al. (1984) *Agric. Biol. Chem.* 48:1763-1775). The effect of the *ae* allele on starch properties has been investigated (Yamada et al. (1978) *Starke* 30:145-148). Increasing doses of *ae* in a *waxy* (*wx*) background produce an increase in the gelatinization temperature so that for the homozygous mutant, incomplete cooking of the starch is observed, even at 95°C. These authors indicate that the increase in viscosity associated with *ae wx* starch is highly desirable and suggest a "target" starch with properties intermediate between *wx* and *ae wx*. While mutations which influence the levels of corn SBEIIa and SBEIIb are available, mutations in the SBEI structural gene have yet to be identified. The lack of SBEI mutants may indicate that the absence of this branching enzyme isoform is lethal to the plant. Alternatively, a SBEI null mutation may give rise to no observable change in seed phenotype or one that is not readily distinguished from existing starch mutants.

Molecular genetic solutions to the generation of starches from corn with altered fine structures have a decided advantage over more traditional plant breeding approaches. Changes to starch fine structure can be produced by specifically inhibiting expression of one or more of the SBE isoforms by antisense inhibition or cosuppression.

function in glucan chain elongation, it is impossible to make predictions concerning starch structure based upon the catalytic properties of each isoform.

SUMMARY OF THE INVENTION

The instant invention discloses utilization of a cDNA clone to construct sense and antisense genes for inhibition of starch branching enzyme enzymatic activity in corn grain or endosperm. More specifically, this invention concerns a method of controlling the branch chain distribution of the amylopectin, the relative proportion of amylose to amylopectin and the degree of polymerization of amylose components of starch in corn comprising: (1) preparing a chimeric gene comprising a nucleic acid fragment encoding a starch branching enzyme structural gene or a fragment thereof, operably linked in either sense or antisense orientation on the upstream side to a nucleic acid fragment that encodes a promoter that directs gene expression in corn endosperm tissue, and operably linked on the downstream side to a nucleic acid fragment encoding a suitable regulatory sequence for transcriptional termination, and (2) transforming corn with said chimeric gene, wherein expression of said chimeric gene results in alteration of the branch chain distribution of the amylopectin molecular component of starch derived from the grain of said transformed corn compared to the branch chain distribution of the amylopectin molecular component of starch derived from corn not possessing said chimeric gene. This invention also concerns corn varieties prepared by transformation using said method, starch isolated from the grain of a corn variety prepared using said method, and a method of preparing a thickened foodstuff comprising combining a foodstuff, water, and an effective amount of a starch isolated from the grain of a corn variety prepared using the said method, and cooking the resulting composition as necessary to produce said thickened foodstuff.

BRIEF DESCRIPTION OF THE DRAWINGS AND SEQUENCE DESCRIPTIONS

The invention can be more fully understood from the following detailed description and the accompanying drawings and the sequence descriptions which form a part of this application.

Figure 1 presents a restriction map of plasmid pBE240 that contains a cDNA insert comprising 78 bp of 5' untranslated DNA, a 2397 bp open reading frame encoding the corn SBEIIb coding region and 190 bp of 3' untranslated DNA.

Figure 2 is a restriction map of plasmid pBE44 comprising a 414 bp 3' fragment of the insert of pBE240 in antisense orientation with respect to the corn 27 kd zein promoter.

Figure 3 is a restriction map of plasmid pML103, used as an intermediate cloning vehicle in construction of chimeric genes of the instant invention.

Figure 15 is a restriction map of plasmid pBE111 comprising a 1810 bp cDNA fragment encoding a truncated SBEI joined in sense orientation with respect to the maize 27 kd zein promoter.

Figure 16 compares RVA profiles of starch from waxy kernels, kernels
5 homozygous for amylose extender (*ae*) and waxy and from kernels containing the pBE44 construct plus waxy. Viscosity, in stirring number units (SNU), and temperature (degrees Celsius) have been measured and plotted as a function of time (in minutes).

SEQ ID NO:1 depicts the nucleotide sequence of the cDNA insert in plasmid pBE240 and the corresponding amino acid sequence of the entire corn SBEIIb enzyme.
10 SEQ ID NO:2 depicts the nucleotide sequence of the the 414 bp insert of pBE44.
SEQ ID NOS:3 and 4 depict the PCR primers BE41 and BE42 used for preparation of the 414 bp insert of pBE44.

SEQ ID NO:5 depicts the nucleotide sequence of the the 507 bp insert of pBE43.
SEQ ID NOS:6 and 7 depict the PCR primers BE39 and BE40 used for
15 preparation of the 507 bp insert of pBE43.

SEQ ID NO:8 depicts the nucleotide sequence of the the 2165 bp insert of pBE45.

SEQ ID NO:9 depicts the nucleotide sequence of the the 2087 bp insert of pBE96.

20 SEQ ID NOS:10 and 11 depict the PCR primers BE14 and BE15 used for preparation of the probe used to isolate the 2772 bp insert of pBE65. BE15 (SEQ ID NO:11) was also used for the preparation of the insert in plasmid pBE79.

SEQ ID NO:12 depicts the nucleotide sequence of the the 2772 bp insert of pBE65.

25 SEQ ID NO:13 depicts the nucleotide sequence of the the 373 bp insert of pBE68.

SEQ ID NOS:14 and 15 depict the PCR primers BE43 and BE52 used for preparation of the 373 bp insert of pBE68.

30 SEQ ID NO:16 depicts the nucleotide sequence of the the 571 bp insert of pBE69.

SEQ ID NOS:17 and 18 depict the PCR primers BE46 and BE50 used for preparation of the 571 bp insert of pBE69.

SEQ ID NO:19 depicts the nucleotide sequence of the the 2487 bp insert of pBE72.

35 SEQ ID NO:20 depicts the nucleotide sequence of the the 1865 bp insert of pBE97.

the repeating crystalline and amorphous units in the starch granule is quite regular with a repeat distance of 9 nm observed in starch from a wide variety of plant species (Jenkins (1993) Starch/Starke 45:417-420). Thus A and B1 chains are less than 9nm in length B2 and B3 chains are between 18 and 27 nm in length and B4+ chains are greater than
5 36 nm.

As used herein, the term "nucleic acid" refers to a large molecule which can be single-stranded or double-stranded, composed of monomers (nucleotides) containing a sugar, phosphate and either a purine or pyrimidine. A "nucleic acid fragment" is a fraction of a given nucleic acid molecule. In higher plants, deoxyribonucleic acid (DNA)
10 is the genetic material while ribonucleic acid (RNA) is involved in the transfer of the information in DNA into proteins. A "genome" is the entire body of genetic material contained in each cell of an organism. The term "nucleotide sequence" refers to a polymer of DNA or RNA which can be single- or double-stranded, optionally containing synthetic, non-natural or altered nucleotide bases capable of incorporation into DNA or
15 RNA polymers.

As used herein, "essentially similar" refers to DNA sequences that may involve base changes that do not cause a change in the encoded amino acid, or which involve base changes which may alter one or more amino acids, but do not affect the functional properties of the protein encoded by the DNA sequence. It is therefore understood that
20 the invention encompasses more than the specific exemplary sequences. Modifications to the sequence, such as deletions, insertions, or substitutions in the sequence which produce silent changes that do not substantially affect the functional properties of the resulting protein molecule are also contemplated. For example, alteration in the gene sequence which reflect the degeneracy of the genetic code, or which results in the
25 production of a chemically equivalent amino acid at a given site, are contemplated; thus, a codon for the amino acid alanine, a hydrophobic amino acid, may be substituted by a codon encoding another hydrophobic amino acid residue such as glycine, valine, leucine, or isoleucine. Similarly, changes which result in substitution of one negatively charged residue for another, such as aspartic acid for glutamic acid, or one positively charged
30 residue for another, such as lysine for arginine, can also be expected to produce a biologically equivalent product. Nucleotide changes which result in alteration of the N-terminal and C-terminal portions of the protein molecule would also not be expected to alter the activity of the protein. In some cases, it may in fact be desirable to make mutants of the sequence in order to study the effect of alteration on the biological
35 activity of the protein. Each of the proposed modifications is well within the routine skill in the art, as is determination of retention of biological activity of the encoded products.

ribozyme sequences that may increase the efficacy of antisense RNA to block gene expression. "Ribozyme" refers to a catalytic RNA and includes sequence-specific endoribonucleases.

As used herein, suitable "regulatory sequences" refer to nucleotide sequences located upstream (5'), within, and/or downstream (3') to a coding sequence, which control the transcription and/or expression of the coding sequences. These regulatory sequences include promoters, translation leader sequences, transcription termination sequences, and polyadenylation sequences. In artificial DNA constructs, regulatory sequences can also control the transcription and stability of antisense RNA.

"Promoter" refers to a DNA sequence in a gene, usually upstream (5') to its coding sequence, which controls the expression of the coding sequence by providing the recognition for RNA polymerase and other factors required for proper transcription. A promoter may also contain DNA sequences that are involved in the binding of protein factors which control the effectiveness of transcription initiation in response to physiological or developmental conditions. It may also contain enhancer elements.

An "enhancer" is a DNA sequence which can stimulate promoter activity. It may be an innate element of the promoter or a heterologous element inserted to enhance the level and/or tissue-specificity of a promoter. "Constitutive" promoters refer to those that direct gene expression in substantially all tissues and demonstrate little temporal or developmental regulation. "Organ-specific" or "development-specific" promoters as referred to herein are those that direct gene expression almost exclusively in specific organs, such as leaves or seeds, or at specific developmental stages in an organ, such as in early or late embryogenesis, respectively.

The term "operably linked" refers to nucleic acid sequences on a single nucleic acid molecule which are associated so that the function of one is affected by the other. For example, a promoter is operably linked with a structural gene (i.e., a gene encoding a starch branching enzyme) when it is capable of affecting the expression of that structural gene (i.e., that the structural gene is under the transcriptional control of the promoter).

The term "expression", as used herein, is intended to mean the production of a functional end-product encoded by a gene. More particularly, "expression" refers to the transcription of the sense (mRNA) or the antisense RNA derived from the nucleic acid fragment(s) of the invention that, in conjunction with the protein apparatus of the cell, results in altered levels of protein product. "Antisense inhibition" refers to the production of antisense RNA transcripts capable of preventing the expression of the target protein. "Overexpression" refers to the production of a gene product in transgenic organisms that exceeds levels of production in normal or non-transformed

A number of genes encoding carbohydrate branching enzymes have been isolated and sequenced. These include glycogen branching enzymes from *Saccharomyces cerevisiae* (Thon et al. (1992) *J. Biol. Chem.* 267:15224-15228), *E. coli* (Baecker et al. (1986) *J. Biol. Chem.* 261:8738-8743), *Bacillus stearothermophilus* (Kiel et al. (1991) *Mol. Gen. Genet.* 230:136-144), *Bacillus caldolyticus* (Kiel et al. (1992) *DNA Seq.* 3: 221-232), human (Thon et al. (1993) *J. Biol. Chem.* 268:7509-7513), *Aspergillus nidulans* (Kiel et al. (1990) *Gene* 89:77-84), *Streptomyces coelicolor* (EMBL accession number X73903), *Streptomyces aurofaciens* (Homerova, D. and Kormanec, J. (1994) *Biochem. Biophys. Acta* 1200:334-336) and starch branching enzymes from corn (Baba et al., (1991) *Biochem. Biophys. Res. Commun.* 181:87-94; Fisher et al. (1993) *Plant Physiol.* 102:1045-1046; Fisher et al. (1995) *Plant Physiol.* 108:1313-1314), pea (Burton et al. (1995) *Plant J.* 7:3-15), potato (Poulsen, P. and Kreiberg, J. D. (1993) *Plant Physiol.* 102:1053-1054), cassava (Salehuzzaman et al. (1992) *Plant Mol. Biol.* 20:809-819), rice (Kawasaki et al. (1993) *Mol. Gen. Genet.* 237:10-16; Mizuno et al. (1993) *J. Biol. Chem.* 268:19084-19091) and *Arabidopsis thaliana* (EMBL accession numbers U18817 and U22428). Preferred among these are the corn starch branching enzyme genes. These genes can be isolated by techniques routinely employed by the skilled artisan for isolation of genes when the nucleotide sequence of the desired gene is known, or when the sequence of a homologous gene from another organism is known.

Sequence information about the desired gene can be used to prepare oligonucleotide probes for identification and isolation of the entire branching enzyme gene from an appropriate genetic library. This library may be a genomic library, wherein the coding region may be contained on a single DNA fragment or may be contained on several distinct DNA fragments. Moreover, two or more exons encoding the branching enzyme may be separated by one or more introns. Alternatively, the library may be a cDNA library wherein the likelihood of isolating a cDNA clone comprising the entire coding region as one contiguous sequence is greater. In either instance, the appropriate clone(s) can be identified by DNA-DNA hybridization with probes corresponding to one or more portions of the desired genes. Alternatively, oligonucleotide primers can be prepared and employed as PCR primers in order to amplify and subsequently isolate all or part of the branching enzyme coding region from genomic DNA, or from the genomic or cDNA libraries described above.

Several different assays can be used to measure branching enzyme activity. In the phosphorylase stimulation assay (Boyer, C. D. and Preiss, J. (1978) *Carbohydr. Res.* 61:321-334), activity is measured indirectly by following the ability of branching enzymes to stimulate formation of α -D-glucan from glucose-1-phosphate by

Promoters utilized to drive gene expression in transgenic plants can be derived from many sources so long as the chosen promoter(s) have sufficient transcriptional activity to accomplish the invention by expressing translatable mRNA or antisense RNA in the desired host tissue. For example, promoters for expression in a wide array of plant organs include those directing the 19S and 35S transcripts in Cauliflower mosaic virus (Odell et al. (1985) *Nature* 313:810-812; Hull et al. (1987) *Virology* 86:482-493), small subunit of ribulose 1,5-bisphosphate carboxylase (Morelli et al. (1985) *Nature* 315:200-204; Broglie et al. (1984) *Science* 224:838-843; Herrera-Estrella et al. (1984) *Nature* 310:115-120; Coruzzi et al. (1984) *EMBO J.* 3:1671-1679; Faciotti et al. (1985) *Bio/Technology* 3:241 and chlorophyll a/b binding protein (Lamppa et al. (1986) *Nature* 316:750-752).

Depending upon the application, it may be desirable to select promoters that are specific for expression in one or more organs of the plant. Examples include the light-inducible promoters of the small subunit of ribulose 1,5-bisphosphate carboxylase, if the expression is desired in photosynthetic organs, or promoters active specifically in seeds.

Preferred promoters are those that allow expression specifically in seeds. This may be especially useful, since seeds are the primary location of long-term starch accumulation. In addition, seed-specific expression may avoid any potential deleterious effects that branching enzyme modulation may have on non-seed organs. Examples of seed-specific promoters include, but are not limited to, the promoters of seed storage proteins. The seed storage proteins are strictly regulated, being expressed almost exclusively in seeds in a highly organ-specific and stage-specific manner (Higgins et al. (1984) *Ann. Rev. Plant Physiol.* 35:191-221; Goldberg et al. (1989) *Cell* 56:149-160; Thompson et al. (1989) *BioEssays* 10:108-113). Moreover, different seed storage proteins may be expressed at different stages of seed development.

There are currently numerous examples for seed-specific expression of seed storage protein genes in transgenic plants. These include genes from monocotyledonous plants such as for barley β -hordein (Marris et al. (1988) *Plant Mol. Biol.* 10:359-366) and wheat glutenin (Colot et al. (1987) *EMBO J.* 6:3559-3564). Moreover, promoters of seed-specific genes, operably linked to heterologous coding sequences in chimeric gene constructs, also maintain their temporal and spatial expression pattern in transgenic plants. Such examples include linking either the Phaseolin or Arabidopsis 2S albumin promoters to the Brazil nut 2S albumin coding sequence and expressing such combinations in tobacco, Arabidopsis, or *Brassica napus* (Altenbach et al. (1989) *Plant Mol. Biol.* 13:513-522; Altenbach et al. (1992) *Plant Mol. Biol.* 18:235-245; De Clercq et al. (1990) *Plant Physiol.* 94:970-979), bean lectin and bean β -phaseolin promoters to

Introns isolated from the maize Adh-1 and Bz-1 genes (Callis et al. (1987) *Genes Dev.* 1:1183-1200), and intron 1 and exon 1 of the maize Shrunken-1 (sh-1) gene (Maas et al. (1991) *Plant Mol. Biol.* 16:199-207) may also be of use to increase expression of introduced genes. Results with the first intron of the maize alcohol dehydrogenase (Adh-1) gene indicate that when this DNA element is placed within the transcriptional unit of a heterologous gene, mRNA levels can be increased by 6.7-fold over normal levels. Similar levels of intron enhancement have been observed using intron 3 of a maize actin gene (Luehrsen, K. R. and Walbot, V. (1991) *Mol. Gen. Genet.* 225:81-93). Enhancement of gene expression by Adh1 intron 6 (Oard et al. (1989) *Plant Cell Rep* 8:156-160) has also been noted. Exon 1 and intron 1 of the maize sh-1 gene have been shown to individually increase expression of reporter genes in maize suspension cultures by 10 and 100-fold, respectively. When used in combination, these elements have been shown to produce up to 1000-fold stimulation of reporter gene expression (Maas et al. (1991) *Plant Mol. Biol.* 16:199-207).

Any 3' non-coding region capable of providing a polyadenylation signal and other regulatory sequences that may be required for proper expression can be used to accomplish the invention. This would include the 3' end from any storage protein such as the 3' end of the 10kd, 15kd, 27kd and alpha zein genes, the 3' end of the bean phaseolin gene, the 3' end of the soybean b-conglycinin gene, the 3' end from viral genes such as the 3' end of the 35S or the 19S cauliflower mosaic virus transcripts, the 3' end from the opine synthesis genes, the 3' ends of ribulose 1,5-bisphosphate carboxylase or chlorophyll a/b binding protein, or 3' end sequences from any source such that the sequence employed provides the necessary regulatory information within its nucleic acid sequence to result in the proper expression of the promoter/coding region combination to which it is operably linked. There are numerous examples in the art that teach the usefulness of different 3' non-coding regions (for example, see Ingelbrecht et al. (1989) *Plant Cell* 1:671-680).

Various methods of introducing a DNA sequence (i.e., of transforming) into eukaryotic cells of higher plants are available to those skilled in the art (see EPO publications 0 295 959 A2 and 0 138 341 A1). Such methods include high-velocity ballistic bombardment with metal particles coated with the nucleic acid constructs (see Klein et al. (1987) *Nature* (London) 327:70-73, and see U.S. Pat. No. 4,945,050), as well as those based on transformation vectors based on the Ti and Ri plasmids of *Agrobacterium spp.*, particularly the binary type of these vectors. Ti-derived vectors transform a wide variety of higher plants, including monocotyledonous and dicotyledonous plants, such as soybean, cotton and rape (Pacciotti et al. (1985)

particular genes. U. S. Pat. Nos. 5,190,931, 5,107,065 and 5,283,323 have taught the feasibility of these techniques, but it is well known that their efficiency is unpredictable. In either case, in order to save time, the person skilled in the art will make multiple genetic constructs containing one or more different parts of the gene to be suppressed, since the art does not teach a method to predict which will be most effective for a particular gene. Furthermore, even the most effective constructs will give an effective suppression phenotype only in a fraction of the individual transgenic lines isolated. For example, WO93/11245 and WO94/11516 teach that when attempting to suppress the expression of fatty acid desaturase genes in canola, actual suppression was obtained in less than 1% of the lines tested. In other species the percentage is somewhat higher, but in no case does the percentage reach 100.

This should not be seen as a limitation on the present invention, but instead as practical matter that is appreciated and anticipated by the person skilled in this art. Accordingly, skilled artisan will develop methods for screening large numbers of transformants. The nature of these screens will generally be chosen on practical grounds, and is not an inherent part of the invention. In the instant case, for example, one can screen by looking for changes in starch phenotype using chromatography to determine relative proportions of amylose to amylopectin, amylopectin branch chain distribution, RVA analysis (as is done in the examples), or other means. One could equally use antibodies specific for the protein encoded by the gene being suppressed, or one could establish assays that specifically measure enzyme activity. A preferred method will be one which allows large numbers of samples to be processed rapidly, since it will be expected that the majority of samples will be negative.

Plants that are identified to have the altered starch fine structure in the grain present unique genetic material which provide advantages over traditional corn lines and known starch mutants. Use of lines with inhibited expression of SBE isoforms in corn breeding provide a dominant trait that can simplify and speed the breeding process. Known starch mutants can be used but they are often recessive and present more complications. Further, the use of antisense or cosuppression to inhibit SBE isoforms leads to variable levels of inhibition due to chromosomal position effects. The resulting variable levels of SBE activities would lead to a wide range of phenotypes that is not possible using traditional mutants which can result in a limited dosage series of a mutant allele in corn endosperm. Additional unique and potentially valuable starch fine structures will result from crossing the newly developed corn lines with inhibited SBE with each other and/or known starch mutants such as *wx* or *ae*.

Cloning sites (NcoI or SmaI) were incorporated into the oligonucleotides to provide antisense orientation of the DNA fragments when inserted into the digested vector pML103 as described below. Amplification was performed in a 100 ml volume in a standard PCR mix consisting of 0.4 mM of each oligonucleotide and 0.3 pM of pBE240 in 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.001% w/v gelatin, 200 mM dGTP, 200 mM dATP, 200 mM dTTP, 200 mM dCTP and 0.025 unit Amplitaq™ DNA polymerase. Reactions were carried out in a Perkin-Elmer Cetus Thermocycler™ for 30 cycles comprising 1 minute at 95°C, 2 minutes at 55°C and 3 minutes at 72°C, with a final 7 minute extension at 72°C after the last cycle. The amplified DNA was digested with restriction enzymes NcoI and SmaI and fractionated on a 0.7% low melting point agarose gel in 40 mM Tris-acetate, pH 8.5, 1 mM EDTA. The appropriate band was excised from the gel, melted at 68°C and combined with a 4.9 kb NcoI-SmaI fragment of the plasmid pML103 (Figure 3). Plasmid pML103 has been deposited under the terms of the Budapest Treaty at ATCC (American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852), and bears the following accession number: ATCC 97366. The DNA segment from pML103 contains a 1.05 kb SalI-NcoI promoter fragment of the maize 27 kD zein gene and a 0.96 kb SmaI-SalI fragment from the 3' end of the maize 10 kD zein gene in the vector pGem9Zf(+) (Promega). Vector and insert DNA were ligated at 15°C overnight, essentially as described (Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning, Cold Spring Harbor Laboratory Press, New York; hereinafter "Maniatis"). The ligated DNA was used to transform *E. coli* XL1-Blue (Epicurian Coli XL-1 Blue™; Stratagene). Bacterial transformants were screened by restriction enzyme digestion of plasmid DNA and limited nucleotide sequence analysis using the dideoxy chain termination method (Sequenase™ DNA Sequencing Kit; U. S. Biochemical). The resulting plasmid construct, pBE44, comprises a chimeric gene encoding, in the 5' to 3' direction, the maize 27 kD zein promoter, a 3' fragment of the corn SBEIIb cDNA, and the 10 kD zein 3' region.

Larger quantities of pBE44 plasmid DNA were prepared by the alkaline lysis method, followed by purification by CsCl density gradient centrifugation.

Transformation of Corn with the 3' Antisense Construct

Immature corn embryos were dissected from developing caryopses derived from crosses of the inbred corn lines H99 and LH132. The embryos were isolated 10 to 11 days after pollination when they were 1.0 to 1.5 mm long. The embryos were placed with the axis-side facing down and in contact with agarose-solidified N6 medium (Chu et al. (1975), *Sci. Sin. Peking* 18:659-668). The embryos were kept in the dark at 27°C.

continued to grow slowly on this medium. After an additional 2 weeks the tissue was transferred to fresh N6 medium containing glufosinate. After 6 weeks, areas of about 1 cm in diameter of actively growing callus were identified on some of the plates containing the glufosinate-supplemented medium. These calli continued to grow when sub-cultured on the selective medium.

Plants were regenerated from the transgenic callus by first transferring clusters of tissue to N6 medium supplemented with 0.2 mg per liter of 2,4-D. After two weeks the tissue was transferred to regeneration medium (Fromm et al. (1990) *Bio/Technology* 8:833-839). A total of 9 corn plants were regenerated from a single transformation experiment using the pBE44 construct.

Molecular Analysis of Transgenic Corn Plants Containing the 3' Antisense Construct

Total DNA was isolated from leaf tissue of plants regenerated from the transformation experiment using pBE44 essentially as described by Dellaporta et al. (Dellaporta et al. (1983) *Plant Mol. Biol. Rep.* 1 (4):9). Lyophilized tissue was frozen in liquid nitrogen, ground to a fine powder and suspended in a buffer consisting of 100 mM Tris-HCl, pH 8.0, 50 mM EDTA, 10 mM β -mercaptoethanol and 0.5 M NaCl. Cells were lysed by the addition of SDS to 1% and the DNA precipitated with isopropanol. The dissolved DNA was treated with DNase-free RNase and then re-precipitated with iso-propanol. The isolated DNAs were dissolved in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA and stored at -20°C until use.

For Southern blot analysis, 5 mg of isolated DNA was digested with restriction enzyme (10 units/mg DNA) in the appropriate buffer for approximately 6 hrs at 37°C. The restricted DNA was loaded onto a 0.8% agarose gel in Tris-borate-EDTA buffer (Maniatis) and electrophoresed at 40 V overnight. Following denaturation and neutralization, the DNA was transferred to an Immobilon™ membrane (Millipore Corporation) using 10X SSC. The Immobilon™ membrane was pre-hybridized at 65°C in an aqueous buffer system consisting of 6X SSPE, 5X Denhardt's reagent, 0.5% SDS and 100 mg/mL denatured salmon sperm DNA as described (Maniatis). The SBE fragment of pBE44 was labeled by nick translation (BRL Nick Translation Kit) and added to the above buffer supplemented with 5% dextran sulfate at a level of $1-2 \times 10^6$ cpm/ml. Hybridization was allowed to proceed at 65°C for 18 h. The membrane was sequentially washed with 2X SSC, 0.1% SDS for 15 minutes at room temperature, 1X SSC, 0.1% SDS for 15 minutes at room temperature and 0.1X SSC, 0.5% SDS for 15 minutes at 50°C. Washed membranes were exposed to Dupont Reflection™ film with an intensifying screen at -80°C.

using a paint mixer, and allowed to settle for 30 minutes. Two mL of 100 mM NaCl was sprayed onto the solution, allowed to settle for 30 minutes, and the protein-toluene layer was aspirated off. The toluene wash step was repeated. Twelve mL water was added and shaken in a paint shaker for 45 seconds. This solution was centrifuged for
5 10 minutes and the water was removed. The water wash was repeated, followed by a final wash with 12 mL of acetone. After shaking and centrifugation steps, the acetone was drained and allowed to evaporate for 1 h. Starch extracts were incubated in a 40°C oven overnight.

Extracted starches were enzymatically debranched as follows. Extracted starches
10 (10 mg) from individual seeds were gelatinized in 2 mL water by heating to 115°C for 0.5 h. Four units of isoamylase (Sigma) in 50 mM NaOAc buffer, pH 4.5, were added to each of the gelatinized starches and placed in a water bath at 45°C for 2.5 h. Enzyme was inactivated by heating samples to 115°C for 5 minutes. Each sample was filtered through a 0.45 micron filter, and placed into individual autosampler vials. Samples were
15 held at 45°C until injection.

Fifty mL of debranched starch sample were injected and run through four columns (3 x 250 Å and 1 x 500 Å ultrahydrogel™; Waters) arranged in series at 45°C and eluted with 50 mM NaOAc at a flow rate of 0.7 mL/min. Sampling interval was 65 minutes. A refractive index detector (Waters), integrator/plotter (Spectra-Physics)
20 and computer were used for sample detection, recording of retention times and chromatogram storage, respectively. Retention times of collected samples were compared to retention times of pullulan standards (380K, 100K, 23.7K, 5.8K, 728 and 180 mw).

Spectra-Physics software was used to make any baseline corrections to the
25 chromatogram including subtraction of a blank chromatogram. Spectra-Physics GPC-PC software was used to enter molecular weights and retention times of pullulan standards. The data were imported to Microsoft Excel for parsing and stripping of all data except molecular weight and area percent of the chromatogram. The remaining data were used to determine branch chain distribution of the amylopectin using Jandel Scientific Peakfit
30 software. A series of six Gaussian curves were fit to the amylopectin portion of the chromatograms as described by Ong et al. ((1994) *Carbohydrate Res.* 260:99-117).

Amylopectin is typically described by its distribution of branch chains in the molecule. The amylopectin molecule is comprised of alternating crystalline and amorphous regions. The crystalline region is where many of the branch points (α -1,6
35 linkages) occur, while the amorphous region is an area of little to no branching and few branch chains. The type of chain is designated A or B. A chains are unbranched and

the antisense gene relative to control starch. The instant transgenic plants thus demonstrate a unique starch branching phenotype compared to non-transgenic control plants. This data indicates that alteration of corn starch branching enzyme activity by suppressing expression of the corresponding genes encoding starch branching enzymes results in an altered starch phenotype.

R1 kernels from the pBE44 line, 0693, were planted and R2 grain was produced. Individual R2 kernels were analyzed using the same procedure as described above for analysis of R1 kernels. Individual kernels from a negative control line (04659, which has been through the transformation process but does not carry the antisense gene) were included in this set of assays. Table 2 presents the results for R2 kernels. The data represent the percentage difference of the various branches between R2 kernels and kernels from the negative control.

Table 2. Percentage Difference of Branch Chain Distribution of Amylopectin From Starch Isolated From Individual R2 Seed From 3' Antisense SBEIIb Transgenic Corn Line (05985) Compared to Starch Isolated From Negative Control Line (04659).

<u>Starch Source</u>	<u>A + B1</u>	<u>B2</u>	<u>B3</u>	<u>B4+</u>
059852	69	91	132	476
0598510	71	92	129	455

As can be seen, long chains (B3 and B4+) are favored at the expense of shorter chains (A's, B1's and B2's) in the amylopectin derived from R2 kernels possessing the antisense gene relative to control starch (04659). The instant transgenic plant thus demonstrates a unique starch branching phenotype compared to non-transgenic control plants. This data also indicates that the phenotype observed in the R2 seed is stronger than that of the R1 seed (Table 1) which may be due to segregation.

R4 grain (line XAY00681) was produced, harvested and starch was extracted. For starch branch chain distribution and determination of amylose content, starch digestion was modified from that in previous examples slightly as follows. Seven mg of each starch sample was added to a screw cap test tube with 1.1 mL of water. The tubes were heated to 120°C for 30 minutes and then placed in a water bath at 45°C. Debranching solution was made by diluting 50 µL of isoamylase (5×10^6 units/mL, Sigma) per mL of sodium acetate buffer (50 mM, pH 4.5). 40 µL of debranching solution was added to each starch sample and incubated for 3 h at 45°C. Reactions were stopped by heating to 110°C for 5 minutes. Debranched starch samples were lyophilized and redissolved in DMSO for analysis by gel permeation chromatography (GPC). One hundred µL of debranched starch was injected and run through 2 columns (Polymer

21-24	4.72	0.18	3.74	0.05
18-21	6.01	0.03	4.83	0.10
15-18	8.42	0.05	6.18	0.12
13-15	7.24	0.21	5.34	0.11
11-15	6.64	0.17	4.49	0.10
9-11	6.20	0.08	4.54	0.11
7-9	4.48	0.06	3.40	0.07
5-7	3.67	0.07	2.91	0.05

Table 4. Percentage Difference of Branch Chain Distribution of Amylopectin (expressed as A+B1, B2, B3 and B4+) and Amylose Content (% of Total Starch) from Starch Isolated from R4 Grain containing the 3' Antisense Transcript of Corn SBEIIb (XAY00681) as Compared to Control (Dent). DP range is indicated.

<u>A+B1 (5-15)</u>	<u>B2 (15-32)</u>	<u>B3 (32-60)</u>	<u>B4+ (60-150)</u>	<u>Amylose (>150)</u>
83.3	89.0	117.4	184.5	128.4

As can be seen in Tables 3 and 4, the relative amount of amylose increased as did the proportion of longer branches on amylopectin in starch which contained the 3' antisense transcript of corn SBE IIb compared to a dent control.

Functional Analysis of Starch from Lines Homozygous for the 3' Antisense Construct

Kernels of plants of a line (XAT00025), homozygous for the pBE44 construct, were isolated from the progeny of line 05985 in order to obtain sufficient quantities of starch for functionality testing. Starch was extracted from dry mature kernels from line XAT00025, dent, and *ae* corn. For each line 15 g of kernels were weighed into a 50 mL Erlenmeyer flask and steeped in 50 mL of steep solution (same as above) for 18 h at 52°C. The kernels were drained and rinsed with water. The kernels were then homogenized using a 20 mm Polytron probe (Kinematica GmbH, Kriens-Luzern, Switzerland) in 50 mL of cold 50 mM NaCl. The homogenate was filtered through a 72 micron mesh screen. The filtrate was brought up to a total volume of 400 mL with 50 mM NaCl and an equal volume of toluene was added. The mixture was stirred with a magnetic stir bar for 1 h at sufficient speed to completely emulsify the two phases. The emulsion was allowed to separate overnight in a covered beaker. The upper toluene layer was aspirated from the beaker and discarded. The starch slurry remaining in the bottom of the beaker was resuspended, poured into a 250 mL centrifuge bottle and centrifuged 15 minutes at 25,000 RCF. The supernatant was discarded and the starch was washed sequentially with water and acetone by shaking and centrifuging as above.

Thermocycler™ for 30 cycles comprising 1 minute at 95°C, 2 minutes at 55°C and 3 minutes at 72°C, with a final 7 minute extension at 72°C after the last cycle. The amplified DNA was digested with restriction enzymes NcoI and SmaI and fractionated on a 0.7% low melting point agarose gel in 40 mM Tris-acetate, pH 8.5, 1 mM EDTA. The appropriate band was excised from the gel, melted at 68°C and combined with a 4.9 kb NcoI-SmaI fragment of the plasmid pML103 (Figure 3). The DNA segment from pML103 contains a 1.05 kb SalI-NcoI promoter fragment of the maize 27 kD zein gene and a 0.96 kb SmaI-SalI fragment from the 3' end of the maize 10 kD zein gene in the vector pGem9Zf(+) (Promega). Vector and insert DNA was ligated at 15°C overnight, essentially as described (Maniatis). The ligated DNA was used to transform *E. coli* XL1-Blue (Epicurian Coli XL-1 Blue'; Stratagene™). Bacterial transformants were screened by restriction enzyme digestion of plasmid DNA and limited nucleotide sequence analysis using the dideoxy chain termination method (Sequenase™ DNA Sequencing Kit; U. S. Biochemical). The resulting plasmid construct, pBE43, comprises a chimeric gene encoding in the 5' to 3' direction, the maize 27 kD zein promoter, a 5' fragment of the corn SBEIIb gene in antisense orientation, and the 10 kD zein 3' region.

Larger quantities of pBE43 plasmid DNA were prepared by the alkaline lysis method, followed by purification by CsCl density gradient centrifugation.

Transformation of Corn with the 5' Antisense Construct

The 5' antisense construct (pBE43) was introduced into embryogenic corn tissue by the particle bombardment method essentially as described in Example 1. Seven days after bombardment the tissue was transferred to N6 medium that contained glufosinate (2 mg per liter) and lacked casein or proline. The tissue continued to grow slowly on this medium. After an additional 2 weeks the tissue was transferred to fresh N6 medium containing glufosinate. After 6 weeks, areas of about 1 cm in diameter of actively growing callus were identified on some of the plates containing the glufosinate-supplemented medium. These calli continued to grow when sub-cultured on the selective medium.

Plants were regenerated from the transgenic callus by first transferring clusters of tissue to N6 medium supplemented with 0.2 mg per liter of 2,4-D. After two weeks the tissue was transferred to regeneration medium (Fromm et al. (1990) *Bio/Technology* 8:833-839). Ninety-nine transgenic plant lines were generated from 2 separate particle bombardment experiments performed with the DNA construct pBE43.

Molecular Analysis of Transformed Corn Plants Containing the 5' Antisense Construct

Southern blot and Northern blot analyses of DNA and RNA from corn plants transformed with the 5' antisense construct (pBE43) were performed as described in

species. This result is consistent with the identity of the 600 base RNA of the antisense transcript of pBE43.

Analysis of Starch from Transformed Corn Plants Containing the 5' Antisense Construct

Starches from individual R1 kernels of plants transformed with pBE43 (the 5' antisense construct for corn SBEIIb) were extracted and analyzed using the procedure described in Example 1. As known to those skilled in the art, the antisense or cosuppression phenomenon is generally not observed in every individual transgenic line. Therefore, individual kernels from multiple lines were examined. No alterations in starch branch chain distribution were observed for the transgenic lines that were screened. It is believed that the number of lines tested was too small to insure finding a plant where an effective antisense event occurred. As described above, the number of plants that must be screened can be unpredictable and large. It is assumed that if a sufficiently large number of individuals were examined such an event would be detected. It may be that this particular configuration is less efficient for suppressing expression of this gene; it is for this reason that multiple constructs were prepared and tested.

EXAMPLE 3

Preparation of Transgenic Corn Expressing a Near Full Length Antisense Transcript of Corn Starch Branching Enzyme IIb

Preparation of the Expression Vector Encoding the Near Full Length Antisense Construct

The construct pBE45 is similar to pBE43 and pBE44 except that the SBEIIb fragment is 2.16 kb and contains the entire 5' untranslated region as well as 2.08 kb of the coding region (SEQ ID NO:8). pBE240 was first digested with EcoRI and then subjected to an end filling reaction with the Klenow fragment of DNA polymerase I (Maniatis). The blunt-ended DNA was fractionated on a low melting point agarose gel and the excised band combined with a 4.9 kb NcoI-SmaI fragment of the plasmid pML103 (Figure 3). The DNA segment from pML103 contains a 1.05 kb SalI-NcoI promoter fragment of the maize 27 kD zein gene and a 0.96 kb SmaI-SalI fragment from the 3' end of the maize 10 kD zein gene in the vector pGem9Zf(+) (Promega). Vector and insert DNA were ligated at 15°C overnight, essentially as described (Maniatis). The ligated DNA was used to transform *E. coli* XL1-Blue (Epicurian Coli XL-1 Blue™; Stratagene). Bacterial transformants were screened for the presence of and the orientation of the added DNA by restriction enzyme digestion with KpnI and limited nucleotide sequence analysis using the dideoxy chain termination method (Sequenase™ DNA Sequencing Kit; U. S. Biochemical). According to this analysis, in pBE45, the SBEIIb fragment is present in inverse orientation relative to the 27 kD zein promoter.

Analysis of Starch from Transformed Corn Plants Containing the Near Full Length Antisense Construct

Starches from individual R1 kernels of plants transformed with pBE45 (the near full length antisense construct for corn SBEIIb) were analyzed using the procedure described in Example 1. As known to those skilled in the art, the antisense phenomenon is generally not observed in every individual transgenic line. Therefore, individual kernels from multiple lines were examined and as expected, some, but not all lines possessed kernels demonstrating an altered starch phenotype. Table 5 presents the results for kernels from a transformed corn line which did show a phenotype. The data represent the percentage difference of the various branches between kernels of the transformed line and kernels from a negative control (line 03376, which has been through the transformation process but does not contain the antisense gene).

Table 5. Percentage Difference of Branch Chain Distribution of Amylopectin from Starch Isolated from Individual Seed from Near Full Length Antisense SBEIIb Transgenic Corn Line (9228) Compared to Starch Isolated from Negative Control Line (03376).

<u>Starch Source</u>	<u>A + B1</u>	<u>B2</u>	<u>B3</u>	<u>B4+</u>
92283	92	97	81	192

As can be seen, long chains (B4+) are favored at the expense of shorter chains (A's and B1's, B2's and B3's) in the starch derived from the corn plant possessing the antisense gene relative to control starch (03376). The instant transgenic plant thus demonstrates a unique starch branching phenotype compared to non-transgenic control plants. This data indicates that alteration of corn starch branching enzyme activity by suppressing expression of the corresponding genes encoding starch branching enzymes results in an altered starch phenotype.

EXAMPLE 4

Preparation of Transgenic Corn Expressing a Near Full Length Sense Transcript of Corn Starch Branching Enzyme IIb

Preparation of the Expression Vector Encoding the Near Full Length Sense Construct

Plasmid pBE96 comprises a 2.09 kb fragment of the SBEIIb cDNA (SEQ ID NO:9) joined in the sense orientation to the 27 kD zein promoter and the 10 kD zein 3' end (Figure 8). The SBEIIb fragment commences at the initiating ATG codon of the coding region and terminates 312 bp 5' of the translation termination codon. pBE240 was subjected to site specific mutagenesis (Sculptor™ Mutagenesis Kit, Amersham) to generate an NcoI site at the ATG start site. The mutagenized plasmid was first digested with EcoRI and then rendered blunt-ended by reaction with Klenow. The DNA

amplify this region of the gene by PCR using corn total DNA as the template. A 571 bp 5' fragment was isolated, sequenced and found to be identical to the cDNA over nucleotides 49 to 188. pBE65 was then used as a starting point in the generation of sense and antisense SBEI constructs including pBE68 and pBE97 described below. In the time since these constructs were made and introduced into corn, a second SBEI sequence became available (Fisher et al. (1995) *Plant Physiol.* 108:1313-1314). The 5' terminal 165 bp of pBE65 showed poor agreement with this sequence as it did with the previous SBEI sequence. As a result of subsequent experiments, it is now concluded that pBE65 contains a 165 bp 5' terminal segment that is not related to SBEI but which presumably arose as an artifact during the cloning of corn cDNA. This region is followed by 2607 bp of SBEI cDNA which encodes 42 amino acids of the SBEI transit peptide, the 760 amino acids of the mature SBEI protein and contains 194 bp of 3' untranslated DNA. The plasmid pBE65 has been deposited under the terms of the Budapest Treaty at the ATCC (American Type Tissue Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852) and bears the following accession number: _____.

Preparation of Expression Vectors Encoding SBEI Antisense Constructs

Since it was not known which portions of the cDNA sequence would be most effective in mediating suppression of SBEI expression, three constructs bearing different SBEI fragments in antisense orientation were made. The chimeric gene of plasmid pBE68 (Figure 9) comprises a 3' fragment of the SBEI cDNA in antisense orientation with respect to the maize 27 kD zein promoter that is located 5' to the SBEI fragment, and the 10 kD zein 3' end that is located 3' to the SBEI fragment. The 373 bp SBEI fragment of this construct (SEQ ID NO:13) was obtained by PCR of pBE65 using the oligonucleotide primer pair BE43 (SEQ ID NO:14) and BE52 (SEQ ID NO:15):

BE43 5' -GAATTCCCGGGCCGAACCTCGTTCAAAG-3' (SEQ ID NO:14)

BE52 5' -GAATTCCATGGCGGTGATGAGACACCAAGTC-3' (SEQ ID NO:15)

The chimeric gene of pBE69 (Figure 10) is analogous to that of pBE68 except that the SBEI fragment consists of a 5' portion of the SBEI cDNA. The 571 bp fragment of this construct (SEQ ID NO:16) was obtained by amplification of pBE65 using the primer pair BE46 (SEQ ID NO:17) and BE50 (SEQ ID NO:18):

BE46 5' -GAATTCCATGGCCATCTTATGGTTTGCACC-3' (SEQ ID NO:17)

BE50 5' -GAATTCCTCGGGCATAGCATAGATGACGGC-3' (SEQ ID NO:18)

Example 1. Seven days after bombardment, the tissue was transferred to N6 medium that contained gluphosinate (2 mg per liter) and lacked casein or proline. The tissue continued to grow slowly on this medium. After an additional 2 weeks, the tissue was transferred to fresh N6 medium containing gluphosinate. After 6 weeks, areas of about 1
5 cm in diameter of actively growing callus were identified on some of the plates containing the gluphosinate supplemented medium. These calli continued to grow when sub-cultured on the selective medium.

Plants were regenerated from the transgenic callus by first transferring clusters of tissue to N6 medium supplemented with 0.2 mg per liter of 2,4-D. After two weeks, the
10 tissue was transferred to regeneration medium (Fromm et al. (1990) *Bio/Technology* 8:833-839). Nine transgenic plant lines were regenerated from particle bombardment experiments performed with the DNA construct pBE68, 20 transgenic lines were regenerated from particle bombardments performed with the DNA construct pBE69 and
15 9 transgenic lines were regenerated from particle bombardment experiments performed with the DNA construct pBE72.

Molecular Analysis of Transgenic Corn Plants Containing the SBEI Antisense Constructs

Total DNA was isolated from leaf tissue of transgenic plants essentially as described in Example 1. For Southern blot analysis of pBE68, pBE69 and pBE72 transformants, 10 mg of isolated DNA was digested with the restriction enzyme XbaI at
20 37°C for 6 hrs in the buffer supplied by the manufacturer. The restricted DNAs were electrophoresed at 40 volts overnight on a 0.8 % agarose gel in Tris-phosphate-EDTA buffer (Maniatis) and transferred to Immobilon™ membranes. The blots were pre-hybridized, hybridized with nick translated pBE65 insert, and washed as described in Example 1.

25 Total RNA was isolated from developing (20-22 DAP) kernels of transgenic plants and Northern blots were prepared as described in Example 1. Blots were probed with nick translated pBE65 insert DNA and subsequently washed according to the regimen outlined in Example 1.

Of the 9 transgenic plant lines that were regenerated from particle bombardments with the pBE68 construct, 5 were identified by Southern blot analysis to contain the trait
30 gene. Northern blot analysis showed variable levels of the 2.7 kb SBEI mRNA in 4 of the Southern positive lines. In addition, 2 of these lines contained a 400 base transcript that presumably corresponds to the antisense RNA specified by the chimeric gene of pBE68. Of the 20 transgenic plant lines that were generated from bombardments with
35 pBE69, 8 were found to contain pBE69 DNA. RNA isolated from two of the pBE69 transgenic plant lines showed the presence of the 600 base antisense transcript. Of the 9

Table 6. The Percentage of Total Chromatographic Area within Given Degree of Polymerization (DP) Ranges for Starch Derived from R4 Kernels Containing the 5' Antisense Transcript of Corn SBE I (XAY01414) and Dent Starch (control). Average of 12 individual seed and standard errors of the mean (SE) are provided.

5

<u>DP range</u>	<u>Dent Starch</u>		<u>XAY01414</u>	
	<u>Average</u>	<u>SE</u>	<u>Average</u>	<u>SE</u>
>5k	5.45	0.14	5.92	0.14
3-5k	2.62	0.05	2.58	0.04
1.8-3k	3.03	0.04	2.95	0.08
1.2-1.8k	2.49	0.05	2.66	0.03
0.9-1.2k	1.92	0.04	2.01	0.04
600-900	2.86	0.03	2.94	0.06
400-600	2.78	0.05	3.07	0.04
250-400	2.83	0.05	3.23	0.04
150-250	2.43	0.04	2.97	0.05
90-150	2.38	0.04	3.61	0.06
60-90	4.04	0.08	5.72	0.15
48-60	4.08	0.07	4.94	0.10
40-48	3.95	0.09	4.86	0.04
32-40	4.52	0.13	5.59	0.14
28-32	3.45	0.12	3.58	0.17
24-28	3.69	0.17	4.40	0.08
21-24	4.72	0.18	4.06	0.18
18-21	6.01	0.03	5.64	0.23
15-18	8.42	0.05	6.17	0.16
13-15	7.24	0.21	5.92	0.28
11-15	6.64	0.17	5.33	0.15
9-11	6.20	0.08	4.71	0.13
7-9	4.48	0.06	3.58	0.09
5-7	3.67	0.07	3.44	0.06

21-24	4.72	0.18	4.67	0.18
18-21	6.01	0.03	5.40	0.12
15-18	8.42	0.05	6.64	0.16
13-15	7.24	0.21	5.73	0.22
11-15	6.64	0.17	5.23	0.11
9-11	6.20	0.08	5.27	0.10
7-9	4.48	0.06	4.08	0.09
5-7	3.67	0.07	3.31	0.10

Table 9. Percentage Difference of Branch Chain Distribution of Amylopectin (expressed as A+B1, B2, B3 and B4+) and Amylose Content (% of Total Starch) from Starch Isolated from R4 Kernels containing the 3' Antisense Transcript of Corn SBE I (XAY00013) as Compared to Control (Dent). DP range is indicated.

<u>A+B1 (5-15)</u>	<u>B2 (15-32)</u>	<u>B3 (32-60)</u>	<u>B4+ (60-150)</u>	<u>Amylose (>150)</u>
85.6	95.9	123.1	135.1	106.0

Like the XAY01414 line, the line transformed with the pBE68 construct has alterations in both the amylose and amylopectin fractions of the starch. Amylose content is increased relative to the control and longer chains (B4+ and B3) are increased in the amylopectin. The majority of the increase in amylose content is due to an increase in the Amylose of DP greater than 5000.

The instant transgenic plants thus demonstrate a unique starch branching pattern compared to the control plants. This data indicates that alteration of corn starch branching enzyme activity by suppressing expression of the corresponding genes encoding starch branching enzymes results in an altered starch phenotype.

EXAMPLE 6

Preparation of Transgenic Corn Expressing Sense Transcripts of Corn Starch Branching

Enzyme I

Preparation of the Expression Vector Encoding the Near Full Length Sense Construct

Plasmid pBE97 comprises a 1.87 kb fragment of the SBEI cDNA of pBE65 (SEQ ID NO:20) joined in the sense orientation to the 27 kD zein promoter and the 10 kD zein 3' end (Figure 13). The SBEI fragment encompasses nucleotides 55 through 1919 of the cDNA clone pBE65 and thus contains 117 bp of unknown sequence preceding the remaining 1748 bp of SBEI coding region DNA. This DNA fragment was generated by PCR-mediated site specific mutagenesis to introduce an NcoI site at nucleotide position 53 of the pBE65 sequence. The appropriate nucleotide primers were combined with pBE65 template DNA in a standard PCR reaction defined in Example 1.

pBE85. pBE85 was subjected to partial digestion with PvuII and the 4.7 kb 10 kD zein-SBEI-10 kD zein fragment was inserted into PvuII digested pKS17 (Example 4). The resultant construct designated pBE98, contains 110 bp of unidentified sequence at the 5' end of SBEI cDNA segment. The correct 5' sequence of the SBEI cDNA was obtained by PCR using oligonucleotides BE101 (SEQ ID NO:22) and BB3 (SEQ ID NO:23):

BE101 5' -AACTGCAGAAGGATCCCATGGTGTGCCTCGTGTGCCCC-3' (SEQ ID NO:22)

BB3 5' -GGATGCTTAAATGTGTACC-3' (SEQ ID NO:23)

10

and lambda DNA prepared from plate lysates of a 19 DAP corn endosperm cDNA library (Stratagene) as the template. The 748 bp PCR product was digested with NcoI and SstI to yield a 673 bp fragment. This DNA segment was exchanged with the corresponding region in pBE98 to give pBE110. The construct pBE110 is 7203 bp in length and consists of a 2565 bp segment of SBEI cDNA (SEQ ID NO:24) that includes the entire 823 amino acids of the SBEI coding region and 96 bp of 3' untranslated DNA (Figure 14). The SBEI DNA fragment is preceded by the promoter region of the maize 10 kD zein gene and is followed by the 3' end of the maize 10 kD zein gene.

The truncated sense SBEI construct pBE111 was generated by assembling a shortened SBEI coding region fragment in the vector pBC24. pBC24 is a pSK+ derivative in which the XbaI site has been blunted by reaction with the Klenow fragment of DNA polymerase and ligated to NcoI linkers. pBC24 thus lacks the XbaI site and contains a unique NcoI site in the polylinker region. The 5' SBEI fragment described above was digested with the restriction enzymes NcoI and BamHI and the 694 bp fragment was cloned into NcoI-BamHI digested pBC24. This intermediate was then digested with BamHI and SmaI and ligated to the 1874 bp BamHI-SmaI fragment of pBE83 to yield pBE112. pBE112 was digested with BstEII, reacted with Klenow and then subjected to partial digestion with NcoI. The liberated 1809 bp fragment was cloned into NcoI-partial SmaI digested pBT752. The vector, pBT752 is a derivative of pKS17 described in Example 4 which contains a 27 kD zein-maize high sulfur zein-10 kD zein chimeric gene and lacks the NcoI site at the translational start site of the hygromycin phosphotransferase gene. Analytical digests of the resultant transformants in NovaBlue (Novagen) cells revealed that the 10 kD zein 3' end was removed as a SmaI fragment during the cloning procedure. This 963 bp SmaI segment was thus isolated from pBT752 and inserted into a blunted HindIII site that is located just downstream from BstEII/SmaI junction in the intermediate plasmid, pBE110.5. Transformants were screened by digestion with DraI in order to determine the orientation of the 3' end fragment relative to the chimeric SBEI gene. From this analysis, pBE111 was identified.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT:
 - (A) NAME: E. I. DU PONT DE NEMOURS AND COMPANY
 - (B) STREET: 1007 MARKET STREET
 - (C) CITY: WILMINGTON
 - (D) STATE: DELAWARE
 - (E) COUNTRY: UNITED STATES OF AMERICA
 - (F) POSTAL CODE (ZIP): 19898
 - (G) TELEPHONE: 302-992-4927
 - (H) TELEFAX: 302-773-0164
 - (I) TELEX: 6717325
- (ii) TITLE OF INVENTION: NOVEL STARCHES VIA MODIFICATION OF
EXPRESSION OF STARCH BIOSYNTHESIS
ENZYME GENES
- (iii) NUMBER OF SEQUENCES: 25
- (iv) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: DISKETTE, 3.50 INCH
 - (B) COMPUTER: IBM PC COMPATIBLE
 - (C) OPERATING SYSTEM: WINDOWS 3.1
 - (D) SOFTWARE: MICROSOFT WORD 6.0A
- (v) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (vi) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: 06/009,113
 - (B) FILING DATE: DECEMBER 20, 1995
- (vii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: BRUCE W. MORRISSEY
 - (B) REGISTRATION NUMBER: 30,663
 - (C) REFERENCE/DOCKET NUMBER: BB-1066

GCA	TTG	GTG	GGT	GAC	TTC	AAC	AAC	TGG	GAT	CCA	AAT	GCA	GAT	CGT	ATG	687
Ala	Leu	Val	Gly	Asp	Phe	Asn	Asn	Trp	Asp	Pro	Asn	Ala	Asp	Arg	Met	
		190				195						200				
AGC	AAA	AAT	GAG	TTT	GGT	GTT	TGG	GAA	ATT	TTT	CTG	CCT	AAC	AAT	GCA	735
Ser	Lys	Asn	Glu	Phe	Gly	Val	Trp	Glu	Ile	Phe	Leu	Pro	Asn	Asn	Ala	
	205				210						215					
GAT	GGT	ACA	TCA	CCT	ATT	CCT	CAT	GGA	TCT	CGT	GTA	AAG	GTG	AGA	ATG	783
Asp	Gly	Thr	Ser	Pro	Ile	Pro	His	Gly	Ser	Arg	Val	Lys	Val	Arg	Met	
220				225						230					235	
GAT	ACT	CCA	TCA	GGG	ATA	AAG	GAT	TCA	ATT	CCA	GCC	TGG	ATC	AAG	TAC	831
Asp	Thr	Pro	Ser	Gly	Ile	Lys	Asp	Ser	Ile	Pro	Ala	Trp	Ile	Lys	Tyr	
				240					245					250		
TCA	GTG	CAG	GCC	CCA	GGA	GAA	ATA	CCA	TAT	GAT	GGG	ATT	TAT	TAT	GAT	879
Ser	Val	Gln	Ala	Pro	Gly	Glu	Ile	Pro	Tyr	Asp	Gly	Ile	Tyr	Tyr	Asp	
			255					260					265			
CCT	CCT	GAA	GAG	GTA	AAG	TAT	GTG	TTC	AGG	CAT	GCG	CAA	CCT	AAA	CGA	927
Pro	Pro	Glu	Glu	Val	Lys	Tyr	Val	Phe	Arg	His	Ala	Gln	Pro	Lys	Arg	
		270					275					280				
CCA	AAA	TCA	TTG	CGG	ATA	TAT	GAA	ACA	CAT	GTC	GGA	ATG	AGT	AGC	CCG	975
Pro	Lys	Ser	Leu	Arg	Ile	Tyr	Glu	Thr	His	Val	Gly	Met	Ser	Ser	Pro	
	285					290					295					
GAA	CCG	AAG	ATA	AAC	ACA	TAT	GTA	AAC	TTT	AGG	GAT	GAA	GTC	CTC	CCA	1023
Glu	Pro	Lys	Ile	Asn	Thr	Tyr	Val	Asn	Phe	Arg	Asp	Glu	Val	Leu	Pro	
300				305						310					315	
AGA	ATA	AAA	AAA	CTT	GGA	TAC	AAT	GCA	GTG	CAA	ATA	ATG	GCA	ATC	CAA	1071
Arg	Ile	Lys	Lys	Leu	Gly	Tyr	Asn	Ala	Val	Gln	Ile	Met	Ala	Ile	Gln	
				320				325						330		
GAG	CAC	TCA	TAT	TAT	GGA	AGC	TTT	GGA	TAC	CAT	GTA	ACT	AAT	TTT	TTT	1119
Glu	His	Ser	Tyr	Tyr	Gly	Ser	Phe	Gly	Tyr	His	Val	Thr	Asn	Phe	Phe	
			335					340					345			
GCG	CCA	AGT	AGT	CGT	TTT	GGT	ACC	CCA	GAA	GAT	TTG	AAG	TCT	TTG	ATT	1167
Ala	Pro	Ser	Ser	Arg	Phe	Gly	Thr	Pro	Glu	Asp	Leu	Lys	Ser	Leu	Ile	
		350				355						360				
GAT	AGA	GCA	CAT	GAG	CTT	GGT	TTG	CTA	GTT	CTC	ATG	GAT	GTG	GTT	CAT	1215
Asp	Arg	Ala	His	Glu	Leu	Gly	Leu	Leu	Val	Leu	Met	Asp	Val	Val	His	
	365				370						375					
AGT	CAT	GCG	TCA	AGT	AAT	ACT	CTG	GAT	GGG	TTG	AAT	GGT	TTT	GAT	GGT	1263
Ser	His	Ala	Ser	Ser	Asn	Thr	Leu	Asp	Gly	Leu	Asn	Gly	Phe	Asp	Gly	
380					385					390					395	
ACA	GAT	ACA	CAT	TAC	TTT	CAC	AGT	GGT	CCA	CGT	GGC	CAT	CAC	TGG	ATG	1311
Thr	Asp	Thr	His	Tyr	Phe	His	Ser	Gly	Pro	Arg	Gly	His	His	Trp	Met	
				400					405					410		
TGG	GAT	TCT	CGC	CTA	TTT	AAC	TAT	GGG	AAC	TGG	GAA	GTT	TTA	AGA	TTT	1359
Trp	Asp	Ser	Arg	Leu	Phe	Asn	Tyr	Gly	Asn	Trp	Glu	Val	Leu	Arg	Phe	
			415					420					425			
CTT	CTC	TCC	AAT	GCT	AGA	TGG	TGG	CTC	GAG	GAA	TAT	AAG	TTT	GAT	GGT	1407
Leu	Leu	Ser	Asn	Ala	Arg	Trp	Trp	Leu	Glu	Glu	Tyr	Lys	Phe	Asp	Gly	
		430					435					440				

GAT CAC CAG TAT ATT TCC CGG AAA CAT GAG GAG GAT AAG GTG ATT GTG	2223
Asp His Gln Tyr Ile Ser Arg Lys His Glu Glu Asp Lys Val Ile Val	
700 705 710 715	
TTC GAA AAG GGA GAT TTG GTA TTT GTG TTC AAC TTC CAC TGC AAC AAC	2271
Phe Glu Lys Gly Asp Leu Val Phe Val Phe Asn Phe His Cys Asn Asn	
720 725 730	
AGC TAT TTT GAC TAC CGT ATT GGT TGT CGA AAG CCT GGG GTG TAT AAG	2319
Ser Tyr Phe Asp Tyr Arg Ile Gly Cys Arg Lys Pro Gly Val Tyr Lys	
735 740 745	
GTG GTC TTG GAC TCC GAC GCT GGA CTA TTT GGT GGA TTT AGC AGG ATC	2367
Val Val Leu Asp Ser Asp Ala Gly Leu Phe Gly Gly Phe Ser Arg Ile	
750 755 760	
CAT CAC GCA GCC GAG CAC TTC ACC GCC GAC TGT TCG CAT GAT AAT AGG	2415
His His Ala Ala Glu His Phe Thr Ala Asp Cys Ser His Asp Asn Arg	
765 770 775	
CCA TAT TCA TCC TCG GTT TAT ACA CCA AGC AGA ACA TGT GTC GTC TAT	2463
Pro Tyr Ser Ser Ser Val Tyr Thr Pro Ser Arg Thr Cys Val Val Tyr	
780 785 790 795	
GCT CCA GTG GAG T GATAGCGGGG TACTCGTTGC TGCGCGGCAT GTGTGGGGCT	2516
Ala Pro Val Glu	

GTCGATGTGA GGAAAAACCT TCTTCCAAAA CCGGCAGATG CATGCATGCA TGCTACAATA 2576

AGGTTCTGAT ACTTTAATCG ATGCTGGAAA GCCCATGCAT CTCGCTGCGT TGTCCTCTCT 2636

ATATATATAA GACCTTCAAG GTGTCAATT 2665

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 414 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GACACCTTGA AGGTCTTATA TATATAGAGA GGACAACGCA GCGAGATGCA TGGGCTTTCC	60
AGCATCGATT AAAGTATCAG AACCTTATTG TAGCATGCAT GCATGCATCT GCCGGTTTTG	120
GAAGAAGGTT TTTCTCACA TCGACAGCCC CACACATGCC GCGCAGCAAC GAGTACCCCG	180
CTATCACTCC ACTGGAGCAT AGACGACACA TGTTCTGCTT GGTGTATAAA CCGAGGATGA	240
ATATGGCCTA TTATCATGCG AACAGTCGGC GGTGAAGTGC TCGGCTGCGT GATGGATCCT	300
GCTAAATCCA CCAAATAGTC CAGCGTCGGA GTCCAAGACC ACCTTATACA CCCCAGGCTT	360
TCGACAACCA ATACGGTAGT CAAAATAGCT GTTGTTGCAG TGGAAGTTGA ACAC	414

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 29 base pairs
 - (B) TYPE: nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GAATTCCCGG GACCCGGATT TCGCTCTT

28

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 29 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GAATTCCATG GTCTATAGAG GCTGTACCG

29

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2165 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

AATTCATATT	TTTGCTCAAG	ATGTTGCATT	GCCTGATCAA	ACTCTTGCAT	ACCATGATAC	60
CTAAGATAGT	CTGCATCACC	CAGGTCAAAT	CTTCGACGAC	ATTTGTCATA	ACTGTTGTTA	120
TTCCCTGGAA	TAAACTTACC	ACTTGGAAGT	CTTTCGGGAC	CTCTTGAAA	ATCTATCCAT	180
TCAGGATGTC	CAAACCTATT	TCCCATGAAA	TTAAGATAGC	CCTCTCCTCC	TAAACCCATT	240
GTGATAAGTC	TAATCATCTT	ATGTAATGCT	ATCCCACGAT	CAATGGTAGG	AGTTGAAGGT	300
CTATCGAGGG	CCATGAAATC	ATACATATCC	TTGTCCATCA	ACCAAAACGC	AATAGTCTTG	360
TCGCCGACTA	ATGCTTGATC	ATGACTTTCA	GCATAAGTTA	CACACTTCTC	TAACCACCTC	420
CTATTTGTCA	GTGTGTGCAC	AATATCACCC	ATCTTCCAAG	TTTCATCACT	TTGCTTGAGA	480
AGGTCAATCC	ATTTGTCAGC	CACAGCCATA	TGCATCCGAT	AGTCAAAACC	TACCCACCA	540
TCGTGAACAG	GAAGGGCAAA	TGTAGGCATT	CCACTAACAT	CTTCACCAAT	GGTTACAGCC	600
TCAGGATAAA	GTCCATGAAT	TAGATCATTT	ACCAGCATCA	AGTAAACCAC	TGCATCTACA	660
TCGGTGGCAA	AGCCAAAATA	CTCATTGAAG	TTCCCCGTAA	ATGTTACTTG	TAATCCGTGG	720
TGAGTGTACA	TCATGGAGGT	CACACCATCA	AAACGGAAAC	CATCAAACCT	ATATTCCTCG	780
AGCCACCATC	TAGCATTGGA	GAGAAGAAAT	CTTAAACCTT	CCCAGTTCCC	ATAGTTAAAT	840
AGGCGAGAAT	CCCACATCCA	GTGATGGCCA	CGTGGACCAC	TGTGAAAGTA	ATGTGTATCT	900
GTACCATCAA	AACCATTCAA	CCCATCCAGA	GTATTACTTG	ACGCATGACT	ATGAACCACA	960
TCCATGAGAA	CTAGCAAACC	AAGCTCATGT	GCTCTATCAA	TCAAAGACTT	CAAATCTTCT	1020
GGGGTACCAA	AACGACTACT	TGGCGCAAAA	AAATTAGTTA	CATGGTATCC	AAAGCTTCCA	1080

CTCTATAGAA GAATCCGTTT AGACATTGAT GAACATGAAG GAGGCTTGGA AGCCTTCTCC 480
CGTAGTTATG AGAAGTTTGG ATTTAATGCC AGCGCGGAAG GTATCACATA TCGAGAATGG 540
GCTCCTGGAG CATTTTCTGC AGCATTGGTG GGTGACTTCA ACAACTGGGA TCCAAATGCA 600
GATCGTATGA GCAAAAATGA GTTTGGTGTT TGGGAAATTT TTCTGCCTAA CAATGCAGAT 660
GGTACATCAC CTATTCCTCA TGGATCTCGT GTAAAGGTGA GAATGGATAC TCCATCAGGG 720
ATAAAGGATT CAATTCCAGC CTGGATCAAG TACTCAGTGC AGGCCCCAGG AGAAATACCA 780
TATGATGGGA TTTATTATGA TCCTCCTGAA GAGGTAAAGT ATGTGTTTCA GCATGCGCAA 840
CCTAAACGAC CAAAATCATT GCGGATATAT GAAACACATG TCGGAATGAG TAGCCCGGAA 900
CCGAAGATAA ACACATATGT AAACTTTAGG GATGAAGTCC TCCCAAGAAT AAAAAAATCT 960
GGATACAATG CAGTGCAAAT AATGGCAATC CAAGAGCACT CATATTATGG AAGCTTTGGA 1020
TACCATGTAA CTAATTTTTT TGCGCCAAGT AGTCGTTTTG GTACCCCAAG AGATTTGAAG 1080
TCTTTGATTG ATAGAGCACA TGAGCTTGGT TTGCTAGTTC TCATGGATGT GGTTCATAGT 1140
CATGCGTCAA GTAATACTCT GGATGGGTTG AATGGTTTTG ATGGTACAGA TACACATTAC 1200
TTTCACAGTG GTCCACGTGG CCATCACTGG ATGTGGGATT CTCGCCTATT TAACTATGGG 1260
AACTGGGAAG TTTTAAGATT TCTTCTCTCC AATGCTAGAT GGTGGCTCGA GGAATATAAG 1320
TTTGATGGTT TCCGTTTTGA TGGTGTGACC TCCATGATGT AACTCACCA CGGATTACAA 1380
GTAACATTTA CGGGGAATCT CAATGAGTAT TTTGGCTTTG CCACCGATGT AGATGCAGTG 1440
GTTTACTTGA TGCTGGTAAA TGATCTAATT CATGGACTTT ATCCTGAGGC TGTAACCATT 1500
GGTGAAGATG TTAGTGGAAT GCCTACATTT GCCCTTCCTG TTCACGATGG TGGGGTAGGT 1560
TTTGACTATC GGATGCATAT GGCTGTGGCT GACAAATGGA TTGACCTTCT CAAGCAAAGT 1620
GATGAAACTT GGAAGATGGG TGATATTGTG CACACACTGA CAAATAGGAG GTGGTTAGAG 1680
AAGTGTGTAA CTTATGCTGA AAGTCATGAT CAAGCATTAG TCGGCGACAA GACTATTGCG 1740
TTTTGGTTGA TGGACAAGGA TATGTATGAT TTCATGGCCC TCGATAGACC TTCAACTCCT 1800
ACCATTGATC GTGGGATAGC ATTACATAAG ATGATTAGAC TTATCACAAT GGCTTTAGGA 1860
GGAGAGGGCT ATCTTAATTT CATGGGAAAT GAGTTTGGAC ATCCTGAATG GATAGATTTT 1920
CCAAGAGGTC CGCAAAGACT TCCAAGTGGT AAGTTTATTC CAGGGAATAA CAACAGTTAT 1980
GACAAATGTC GTCGAAGATT TGACCTGGGT GATGCAGACT ATCTTAGGTA TCATGGTATG 2040
CAAGAGTTTG ATCAGGCAAT GCAACATCTT GAGCAAAAAT ATGAATT 2087

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

GAT Asp 100	GTC Val 100	GAC Asp 100	CAT His 100	CTC Leu 100	CCC Pro 105	ATA Ile 105	TAC Tyr 105	GAC Asp 110	CTG Leu 110	GAC Asp 110	CCC Pro 110	AAG Lys 110	CTG Leu 110	GAG Glu 115	ATA Ile 115	393
TTC Phe 120	AAG Lys 120	GAC Asp 120	CAT His 120	TTC Phe 120	AGG Arg 120	TAC Tyr 120	CGG Arg 125	ATG Met 125	AAA Lys 125	AGA Arg 125	TTC Phe 130	CTA Leu 130	GAG Glu 130	CAG Gln 130	AAA Lys 130	441
GGA Gly 135	TCA Ser 135	ATT Ile 135	GAA Glu 135	GAA Glu 135	AAT Asn 135	GAG Glu 140	GGA Gly 140	AGT Ser 140	CTT Leu 140	GAA Glu 145	TCT Ser 145	TTT Phe 145	TCT Ser 145	AAA Lys 145	GGC Gly 145	489
TAT Tyr 150	TTG Leu 150	AAA Lys 150	TTT Phe 150	GGG Gly 150	ATT Ile 150	AAT Asn 155	ACA Thr 155	AAT Asn 155	GAG Glu 155	GAT Asp 160	GGA Gly 160	ACT Thr 160	GTA Val 160	TAT Tyr 160	CGT Arg 160	537
GAA Glu 165	TGG Trp 165	GCA Ala 165	CCT Pro 165	GCT Ala 165	GCG Ala 170	CAG Gln 170	GAG Glu 170	GCA Ala 170	GAG Glu 175	CTT Leu 175	ATT Ile 175	GGT Gly 175	GAC Asp 175	TTC Phe 175	AAT Asn 175	585
GAC Asp 180	TGG Trp 180	AAT Asn 180	GGT Gly 180	GCA Ala 185	AAC Asn 185	CAT His 185	AAG Lys 185	ATG Met 190	GAG Glu 190	AAG Lys 190	GAT Asp 190	AAA Lys 190	TTT Phe 190	GGT Gly 195	GTT Val 195	633
TGG Trp 200	TCG Ser 200	ATC Ile 200	AAA Lys 200	ATT Ile 200	GAC Asp 200	CAT His 200	GTC Val 205	AAA Lys 205	GGG Gly 205	AAA Lys 205	CCT Pro 210	GCC Ala 210	ATC Ile 210	CCT Pro 210	CAC His 210	681
AAT Asn 215	TCC Ser 215	AAG Lys 215	GTT Val 215	AAA Lys 215	TTT Phe 215	CGC Arg 220	TTT Phe 220	CTA Leu 220	CAT His 220	GGT Gly 225	GGA Gly 225	GTA Val 225	TGG Trp 225	GTT Val 225	GAT Asp 225	729
CGT Arg 230	ATT Ile 230	CCA Pro 230	GCA Ala 230	TTG Leu 230	ATT Ile 230	CGT Arg 235	TAT Tyr 235	GCG Ala 235	ACT Thr 235	GTT Val 240	GAT Asp 240	GCC Ala 240	TCT Ser 240	AAA Lys 240	TTT Phe 240	777
GGA Gly 245	GCT Ala 245	CCC Pro 245	TAT Tyr 245	GAT Asp 245	GGT Gly 250	GTT Val 250	CAT His 250	TGG Trp 250	GAT Asp 255	CCT Pro 255	CCT Pro 255	GCT Ala 255	TCT Ser 255	GAA Glu 255	AGG Arg 255	825
TAC Tyr 260	ACA Thr 260	TTT Phe 260	AAG Lys 260	CAT His 265	CCT Pro 265	CGG Arg 265	CCT Pro 270	TCA Ser 270	AAG Lys 270	CCT Pro 270	GCT Ala 270	GCT Ala 270	CCA Pro 275	CGT Arg 275	ATC Ile 275	873
TAT Tyr 280	GAA Glu 280	GCC Ala 280	CAT His 280	GTA Val 280	GGT Gly 280	ATG Met 285	AGT Ser 285	GGT Gly 285	GAA Glu 285	AAG Lys 285	CCA Pro 290	GCA Ala 290	GTA Val 290	AGC Ser 290	ACA Thr 290	921
TAT Tyr 295	AGG Arg 295	GAA Glu 295	TTT Phe 295	GCA Ala 295	GAC Asp 295	AAT Asn 300	GTG Val 300	TTG Leu 300	CCA Pro 300	CGC Arg 300	ATA Ile 305	CGA Arg 305	GCA Ala 305	AAT Asn 305	AAC Asn 305	969
TAC Tyr 310	AAC Asn 310	ACA Thr 310	GTT Val 310	CAG Gln 310	TTG Leu 310	ATG Met 315	GCA Ala 315	GTT Val 315	ATG Met 315	GAG Glu 320	CAT His 320	TCG Ser 320	TAC Tyr 320	TAT Tyr 320	GCT Ala 320	1017
TCT Ser 325	TTC Phe 325	GGG Gly 325	TAC Tyr 325	CAT His 325	GTG Val 325	ACA Thr 330	AAT Asn 330	TTC Phe 330	TTT Phe 330	GCG Ala 335	GTT Val 335	AGC Ser 335	AGC Ser 335	AGA Arg 335	TCA Ser 335	1065

ACT	GGC	ATG	TCA	GAC	TTG	CAG	CCT	GCT	TCA	CCT	ACA	ATT	GAT	CGA	GGG	1833
Thr	Gly	Met	Ser	Asp	Leu	Gln	Pro	Ala	Ser	Pro	Thr	Ile	Asp	Arg	Gly	
580					585					590					595	
ATT	GCA	CTC	CAA	AAG	ATG	ATT	CAC	TTC	ATC	ACA	ATG	GCC	CTT	GGA	GGT	1881
Ile	Ala	Leu	Gln	Lys	Met	Ile	His	Phe	Ile	Thr	Met	Ala	Leu	Gly	Gly	
				600					605					610		
GAT	GGC	TAC	TTG	AAT	TTT	ATG	GGA	AAT	GAG	TTT	GGT	CAC	CCA	GAA	TGG	1929
Asp	Gly	Tyr	Leu	Asn	Phe	Met	Gly	Asn	Glu	Phe	Gly	His	Pro	Glu	Trp	
			615					620					625			
ATT	GAC	TTT	CCA	AGA	GAA	GGG	AAC	AAC	TGG	AGC	TAT	GAT	AAA	TGC	AGA	1977
Ile	Asp	Phe	Pro	Arg	Glu	Gly	Asn	Asn	Trp	Ser	Tyr	Asp	Lys	Cys	Arg	
		630					635					640				
CGA	CAG	TGG	AGC	CTT	GTG	GAC	ACT	GAT	CAC	TTG	CGG	TAC	AAG	TAC	ATG	2025
Arg	Gln	Trp	Ser	Leu	Val	Asp	Thr	Asp	His	Leu	Arg	Tyr	Lys	Tyr	Met	
		645				650					655					
AAT	GCG	TTT	GAC	CAA	GCG	ATG	AAT	GCG	CTC	GAT	GAG	AGA	TTT	TCC	TTC	2073
Asn	Ala	Phe	Asp	Gln	Ala	Met	Asn	Ala	Leu	Asp	Glu	Arg	Phe	Ser	Phe	
660					665					670					675	
CTT	TCG	TCG	TCA	AAG	CAG	ATC	GTC	AGC	GAC	ATG	AAC	GAT	GAG	GAA	AAG	2121
Leu	Ser	Ser	Ser	Lys	Gln	Ile	Val	Ser	Asp	Met	Asn	Asp	Glu	Glu	Lys	
				680					685					690		
GTT	ATT	GTC	TTT	GAA	CGT	GGA	GAT	TTA	GTT	TTT	GTT	TTC	AAT	TTC	CAT	2169
Val	Ile	Val	Phe	Glu	Arg	Gly	Asp	Leu	Val	Phe	Val	Phe	Asn	Phe	His	
			695					700					705			
CCC	AAG	AAA	ACT	TAC	GAG	GGC	TAC	AAA	GTG	GGA	TGC	GAT	TTG	CCT	GGG	2217
Pro	Lys	Lys	Thr	Tyr	Glu	Gly	Tyr	Lys	Val	Gly	Cys	Asp	Leu	Pro	Gly	
		710					715					720				
AAA	TAC	AGA	GTA	GCC	CTG	GAC	TCT	GAT	GCT	CTG	GTC	TTC	GGT	GGA	CAT	2265
Lys	Tyr	Arg	Val	Ala	Leu	Asp	Ser	Asp	Ala	Leu	Val	Phe	Gly	Gly	His	
		725				730					735					
GGA	AGA	GTT	GGC	CAC	GAC	GTG	GAT	CAC	TTC	ACG	TCG	CCT	GAA	GGG	GTG	2313
Gly	Arg	Val	Gly	His	Asp	Val	Asp	His	Phe	Thr	Ser	Pro	Glu	Gly	Val	
740					745					750					755	
CCA	GGG	GTG	CCC	GAA	ACG	AAC	TTC	AAC	AAC	CGG	CCG	AAC	TCG	TTC	AAA	2361
Pro	Gly	Val	Pro	Glu	Thr	Asn	Phe	Asn	Asn	Arg	Pro	Asn	Ser	Phe	Lys	
				760					765					770		
GTC	CTT	TCT	CCG	CCC	CGC	ACC	TGT	GTG	GCT	TAT	TAC	CGT	GTA	GAC	GAA	2409
Val	Leu	Ser	Pro	Pro	Arg	Thr	Cys	Val	Ala	Tyr	Tyr	Arg	Val	Asp	Glu	
			775					780					785			
GCA	GGG	GCT	GGA	CGA	CGT	CTT	CAC	GCG	AAA	CGA	GAG	ACA	GGA	AAG	ACG	2457
Ala	Gly	Ala	Gly	Arg	Arg	Leu	His	Ala	Lys	Arg	Glu	Thr	Gly	Lys	Thr	
		790				795						800				
TCT	CCA	GCA	GAG	AGC	ATC	GAC	GTC	AAA	GCT	TCC	AGA	GCT	AGT	AGC	AAA	2505
Ser	Pro	Ala	Glu	Ser	Ile	Asp	Val	Lys	Ala	Ser	Arg	Ala	Ser	Ser	Lys	
		805				810					815					

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GAATTCCATG GCGGTGATGA GACACCAGTC

30

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 571 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CCATCTTATG GTTTGCACCA TTCCAGTCAT TGAAGTCACC AATAAGCTCT GCCTCCTGCG 60
CAGCAGGTGC CCATTACGA TATACAGTTC CATCCTCATT TGTATTAATC CCAAATTTCA 120
AATAGCCTTT AGAAAAAGAT TCAAGACCTT CCCTCATTTT CTTCAATTGA TCCTTTCTGC 180
TCTAGGAATC TTTTCATCCG GTACCTGAAA TGGTCCTTGA ATATCTCCAG CTTGGGGTCC 240
AGGTCGTATA TGGGGAGATG GTCGACATCG CTTTGGCAG TTGCCATAGT TTTATCTTCT 300
TGCACAGTAG CTGCAGTGGC GAATTTGCTC TTGACCTTCC GCACCCCTGA CCGGCGAGCC 360
TTGCACTGGA CAGACAACAC ACTCAGGCGC ACATTGCCGC CACCCGCGAT CCCC GGCGGT 420
GCCGCCCGAT CAGCATGCCA GCAAGAGCGA CGATGGCCTT GGAGTCGTCG TCGGCCGGCT 480
TGGCGGGCGA CGGTGGCAAC GGCACGACGG CTTTCTCCTC GGCGATGTCC TTGGCGGCCT 540
CCACCTCCGC CGCCGTCATA TCTATGCTAT G 571

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "PCR primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GAATTCCATG GCCATCTTAT GGTTTGCACC

30

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

TACCTCCCAG TTAGCATAGT TGAACAGCCG ACTATCCCAA AGTTTATGAT AACCTCTATC 1260
 TCCCGCATGA AAATAGGACT CTTGGGTGCT TTGTCCAACA TCATAGCCAT TTAAACCATC 1320
 TGTGACATTA TTA CTGTCAT GGCTATGGAC AACATCCATC AGAACTCGCA AACCCAAACT 1380
 GTGTGCCTTA TCAACAAGAT ATTTGAGGTC CTCTGGTGTG CCTGATCTGC TGCTAACCGC 1440
 AAAGAAATTT GTCACATGGT ACCCGAAAGA AGCATAGTAC GAATGCTCCA TAACTGCCAT 1500
 CAACTGAACT GTGTTGTAGT TATTTGCTCG TATGCGTGGC AACACATTGT CTGCAAATTC 1560
 CCTATATGTG CTTACTGCTG GCTTTTCACC ACTCATACCT ACATGGGCTT CATAGATACG 1620
 TGGAGCAGCA GGCTTTGAAG GCCGAGGATG CTTAAATGTG TACCTTTCAG AAGCAGGAGG 1680
 ATCCCAATGA ACACCATCAT AGGGAGCTCC AAATTTAGAG GCATCAACAG TCGCATAACG 1740
 AATCAATGCT GGAATACGAT CAACCCATAC TCCACCATGT AGAAAGCGAA ATTTAACCTT 1800
 GGAATTGTGA GGGATGGCAG GTTTCCTTT GACATGGTCA ATTTTGATCG ACCAAACACC 1860
 AAATTTATCC TTCTCCATCT TATGGTTTGC ACCATTCCAG TCATTGAAGT CACCAATAAG 1920
 CTCTGCCTCC TGCGCAGCAG GTGCCCATT CAGATATACA GTTCCATCCT CATTTGTATT 1980
 AATCCCAAAT TTCAAATAGC CTTTAGAAAA AGATTCAAGA CTTCCCTCAT TTTCTTCAAT 2040
 TGATCCTTTC TGCTCTAGGA ATCTTTTCAT CCGGTACCTG AAATGGTCCT TGAATATCTC 2100
 CAGCTTGGGG TCCAGGTCGT ATATGGGGAG ATGGTCGACA TCGCCTTTGG CAGTTGCCAT 2160
 AGTTTTATCT TCTTGACAG TAGCTGCAGT GGCGAATTG CTCTTGACCT TCCGCACCCC 2220
 TGACCGGCGA GCCTTGCACT GGACAGACAA CACACTCAGG CGCACATTGC CGCCACCCGC 2280
 GATCCCCGGC GGTGCCGCCC GATCAGCATG CGAGCAAGAG CGACGATGGC CTTGGAGTCG 2340
 TCGTCGGCCG GCTTGGCGGG CGACGGTGGC AACGGCACGA CGGCCTTCTC CTCGGCGATG 2400
 TCCTTGGCGG CCTCCACCTC CGCCGCCGTC ATATCTATGC TATGCTACCT GCTGCTGCTG 2460
 CTGCTGAATT CCTCACTCG ATCAGCA 2487

(2) INFORMATION FOR SEQ ID NO:20:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 1865 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

ATGGCGGCGG CGGAGGTGGA GGCCGCCAAG GACATCGCCG AGGAGAAGGC CGTCGTGCCG 60
 TTGCCACCGT CGCCCGCCAA GCCGGCCGAC GACGACTCCA AGGCCATCGT CGCTCTTGCT 120
 CGCATGCTGA TCGGGCGGCA CCGCCGGGGA TCGCGGGTGG CGGCAATGTG CGCCTGAGTG 180

67

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PCR primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

CGCGGATCCC GGGTTCCAAG GCGCCAGCG G

31

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PCR primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

AACTGCAGAA GGATCCCATG GTGTGCCTCG TGTCGCCC

38

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PCR primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

GGATGCTTAA ATGTGTACC

19

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2565 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

ATGGTGTGCC TCGTGTGCC CTCTCCTCG CCGACTCCGC TTCCGCCGCC GCGGCGCTCT 60
CGCTCGCATG CTGATCGGGC GGCACCGCCG GGGATCGCGG GTGGCGGCAA TGTGCGCCTG 120
AGTGTGTTGT CTGTCCAGTG CAAGGCTCGC CGGTCAGGGG TCGGAAGST CAAGAGCAAA 180
TTCCGCACTG CAGCTACTGT GCAAGAAGAT AAAACTATGG CAACTGCCAA AGGCGATGTC 240
GACCATCTCC CCATATACGA CCTGGACCCC AAGCTGAGA TATTCAAGGA CCATTTTCAGG 300

CCTGGGAAAT ACAGAGTAGC CCTGGACTCT GATGCTCTGG TCTTCGGTGG ACATGGAAGA 2160
GTTGGCCACG ACGTGGATCA CTTACGTCG CCTGAAGGGG TGCCAGGGGT GCGCGAAACG 2220
AACTTCAACA ACCGGCCGAA CTCGTTCAAA GTCCTTTCTC CGCCCCGCAC CTGTGTGGCT 2280
TATTACCGTG TAGACGAAGC AGGGGCTGGA CGACGTCTTC ACGCGAAACG AGAGACAGGA 2340
AAGACGTCTC CAGCAGAGAG CATCGACGTC AAAGCTTCCA GAGCTAGTAG CAAAGAAGAC 2400
AAGGAGGCAA CGGCTGGTGG CAAGAAGGGA TGGAAGTTTG CGCGGCAGCC ATCCGATCAA 2460
GATACCAAAT GAAGCCAGGA GTCCTTGGTG AGGACTGGAC TGGCTGCCGG CGCCCTGTTA 2520
GTAGTCCTGC TCTACTGGAC TAGCCGCCGC TGGCGCCCTT GGAAC 2565

(2) INFORMATION FOR SEQ ID NO:25:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1809 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

ATGGTGTGCC TCGTGTGCC CTCTTCCTCG CCGACTCCGC TTCCGCCGCC GCGGCGCTCT 60
CGCTCCGATG CTGATCGGGC GGCACCGCCG GGGATCGCGG GTGGCGGCAA TGTGCGCCTG 120
AGTGTGTTGT CTGTCCAGTG CAAGGCTCGC CGGTCAGGGG TGCGGAAGGT CAAGAGCAAA 180
TTCGCCACTG CAGCTACTGT GCAAGAAGAT AAAACTATGG CAACTGCCAA AGGCGATGTC 240
GACCATCTCC CCATATACGA CCTGGACCCC AAGCTGGAGA TATTCAAGGA CCATTTTCAGG 300
TACCGGATGA AAAGATTCTT AGAGCAGAAA GGATCAATTG AAGAAAATGA GGAAGTCTT 360
GAATCTTTTT CTAAAGGCTA TTTGAAATTT GGGATTAATA CAAATGAGGA TGGAACTGTA 420
TATCGTGAAT GGGCACCTGC TGCGCAGGAG GCAGAGCTTA TTGSTGACTT CAATGACTGG 480
AATGGTGCAA ACCATAAGAT GGAGAAGGAT AAATTTGGTG TTTGGTCGAT CAAAATTGAC 540
CATGTCAAAG GGAAACCTGC CATCCCTCAC AATTCCAAGG TTAAATTTTC CTTTCTACAT 600
GGTGGAGTAT GGGTTGATCG TATTCCAGCA TTGATTCGTT ATGCGACTGT TGATGCCTCT 660
AAATTTGGAG CTCCCTATGA TGGTGTTTCAT TGGGATCCTC CTGCTTCTGA AAGGTACACA 720
TTTAAGCATC CTCGGCCTTC AAAGCCTGCT GCTCCACGTA TCTATGAAGC CCATGTAGGT 780
ATGAGTGGTG AAAAGCCAGC AGTAAGCACA TATAGGGAAT TTGCAGACAA TGTGTTGCCA 840
CGCATACGAG CAAATAACTA CAACACAGTT CAGTTGATGG CAGTTATGGA GCATTCGTAC 900
TATGCTTCTT TCGGGTACCA TGTGACAAAT TTCTTTGCCG TTAGCAGCAG ATCAGGCACA 960

CLAIMS

What is claimed is:

1. A method of controlling the starch fine structure of starch derived from the grain of corn comprising:

- 5 (a) preparing a chimeric gene comprising a nucleic acid fragment encoding a starch branching enzyme structural gene or a fragment thereof, operably linked in either sense or antisense orientation on the upstream side to a nucleic acid fragment encoding a promoter that directs gene expression in corn endosperm tissue, and operably linked on the downstream side to a nucleic acid fragment encoding a suitable regulatory sequence for transcriptional termination,
- 10

(b) transforming corn with the chimeric gene of step (a), wherein expression of said chimeric gene results in alteration of the fine structure of starch derived from the grain of said transformed corn compared to the fine structure of starch derived from corn not possessing said chimeric gene.

15

2. The method of Claim 1 wherein said alteration of starch fine structure comprises alteration of the branch chain distribution of the amylopectin molecular component of said starch.

3. The method of Claim 1 wherein said alteration of starch fine structure comprises alteration of the ratio of the amylose molecular component to the amylopectin molecular component of said starch.

20

4. The method of Claim 1 wherein said alteration of starch fine structure comprises alteration of the degree of polymerization of the amylose molecular component of said starch.

5. The method of Claim 1 wherein said alteration of starch fine structure comprises alteration of the branch chain distribution of the amylopectin molecular component and alteration of the ratio of the amylose molecular component to the amylopectin molecular component of said starch.

25

6. The method of Claim 1 wherein said alteration of starch fine structure comprises alteration of the branch chain distribution of the amylopectin molecular component and alteration of the degree of polymerization of the amylose molecular component of said starch.

30

7. The method of Claim 1 wherein said alteration of starch fine structure comprises alteration of the ratio of the amylose molecular component to the amylopectin molecular component and alteration of the degree of polymerization of the amylose molecular component of said starch.

35

8. The method of Claim 1 wherein said alteration of starch fine structure comprises alteration of the branch chain distribution of the amylopectin molecular

B4+ chains compared to the branch chain distribution of the amylopectin molecular component of starch isolated from the grain of untransformed corn.

18. Starch isolated from the grain of a corn variety prepared by the method of Claim 1 or any progeny thereof.

5 19. A method of preparing a thickened foodstuff comprising combining a foodstuff, water, and an effective amount of a starch of Claim 18 and cooking the resulting composition as necessary to produce said thickened foodstuff.

10 20. A corn variety transformed with a chimeric gene comprising a nucleic acid fragment encoding a starch branching enzyme structural gene or fragment thereof, operably linked in either sense or antisense orientation on the upstream side to a nucleic acid fragment encoding a promoter that directs gene expression in corn endosperm tissue, and operably linked on the downstream side to a nucleic acid fragment encoding a suitable regulatory sequence for transcriptional termination, or any progeny thereof.

15 21. A method of controlling the branch chain distribution of the amylopectin molecular component of starch in corn comprising:

(a) preparing a chimeric gene comprising a nucleic acid fragment encoding a starch branching enzyme structural gene or a fragment thereof, operably linked in either sense or antisense orientation on the upstream side to a nucleic acid fragment encoding a promoter that directs gene expression in corn endosperm tissue, and operably linked on the downstream side to a nucleic acid fragment encoding a suitable regulatory sequence for transcriptional termination,

20 (b) transforming corn with the chimeric gene of step (a), wherein expression of said chimeric gene results in alteration of the branch chain distribution of the amylopectin molecular component of starch derived from the grain of said transformed corn compared to the branch chain distribution of the amylopectin molecular component of starch derived from corn not possessing said chimeric gene.

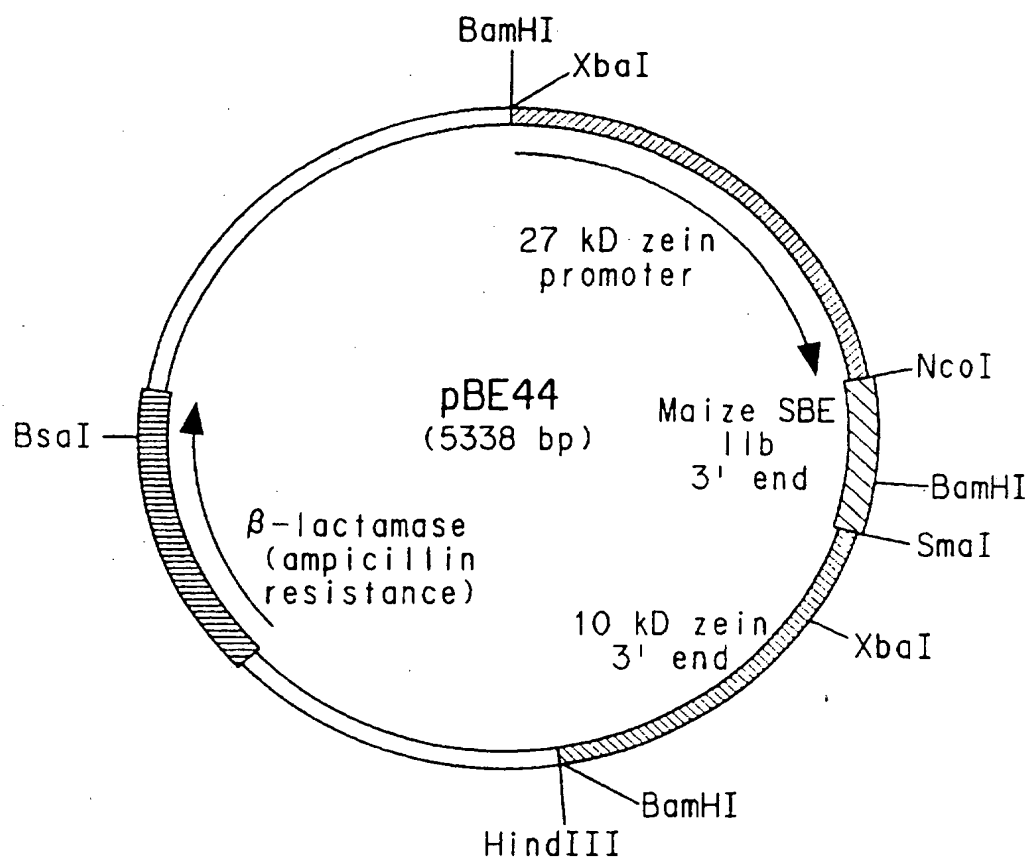


FIG.2

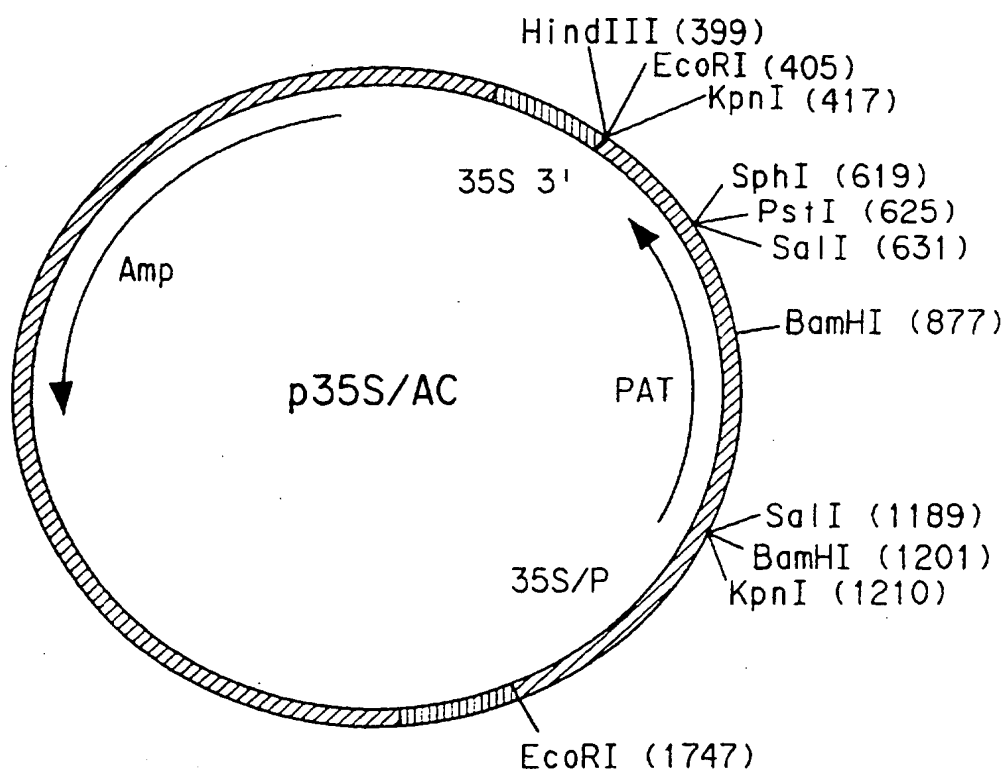


FIG. 4

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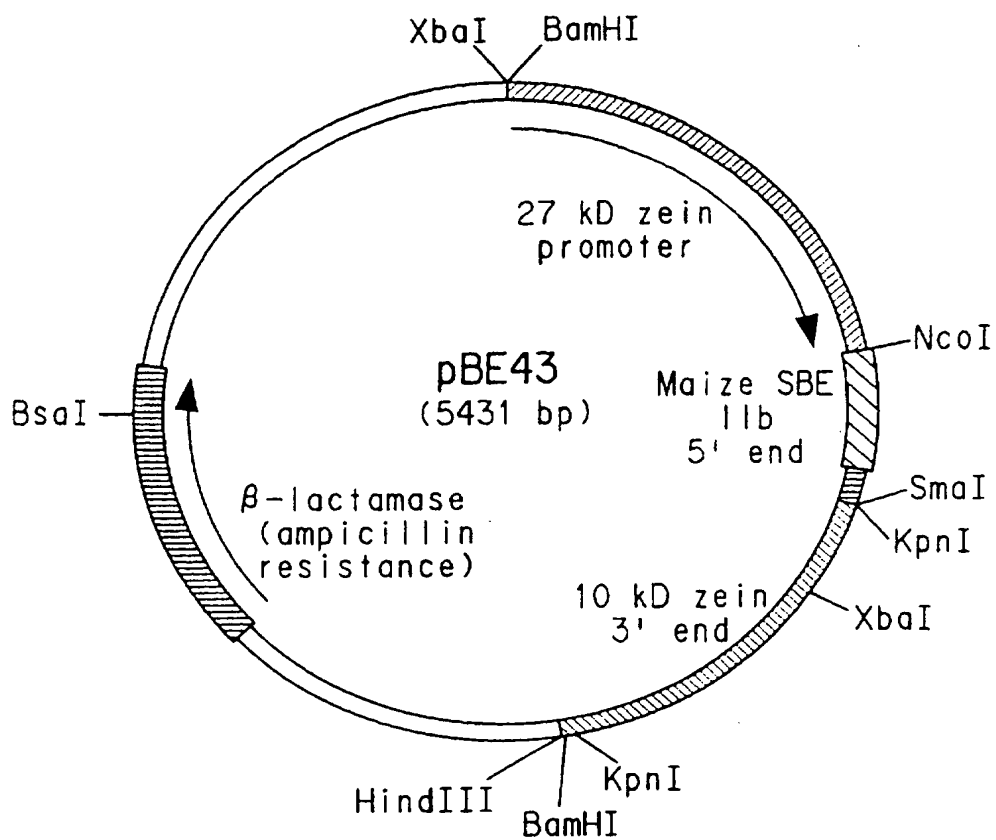


FIG.6

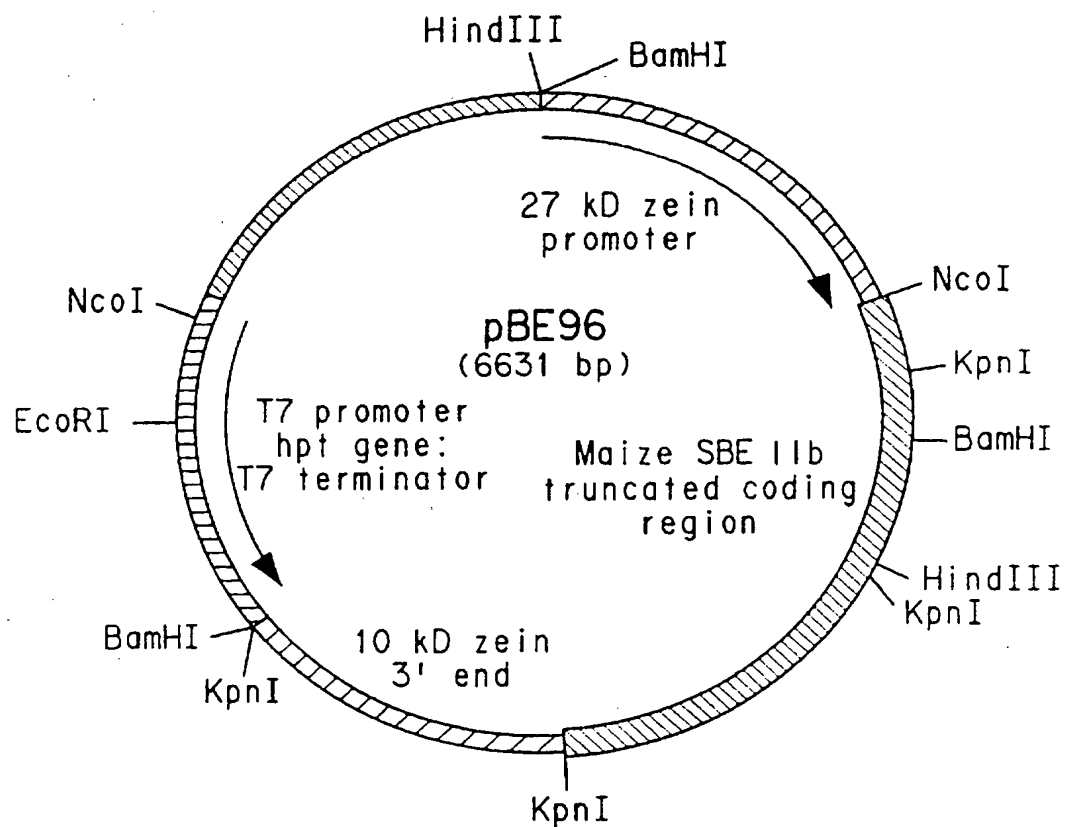


FIG.8

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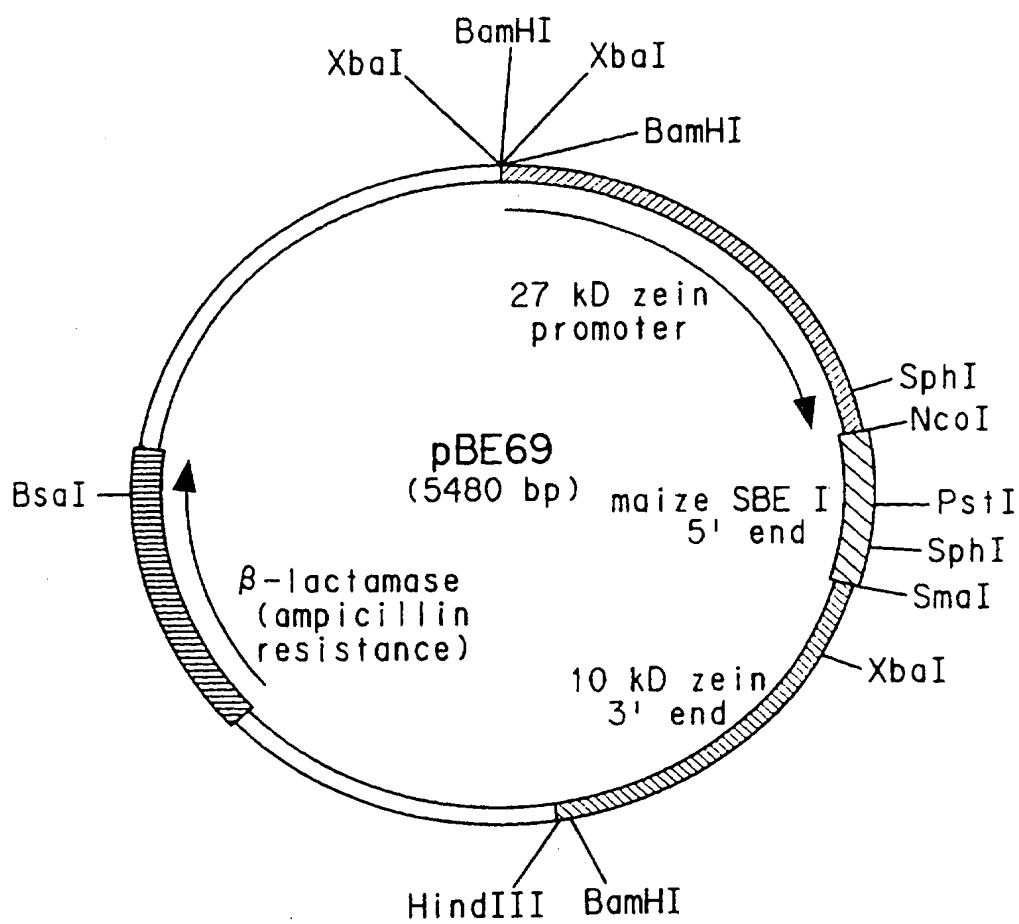


FIG. 10

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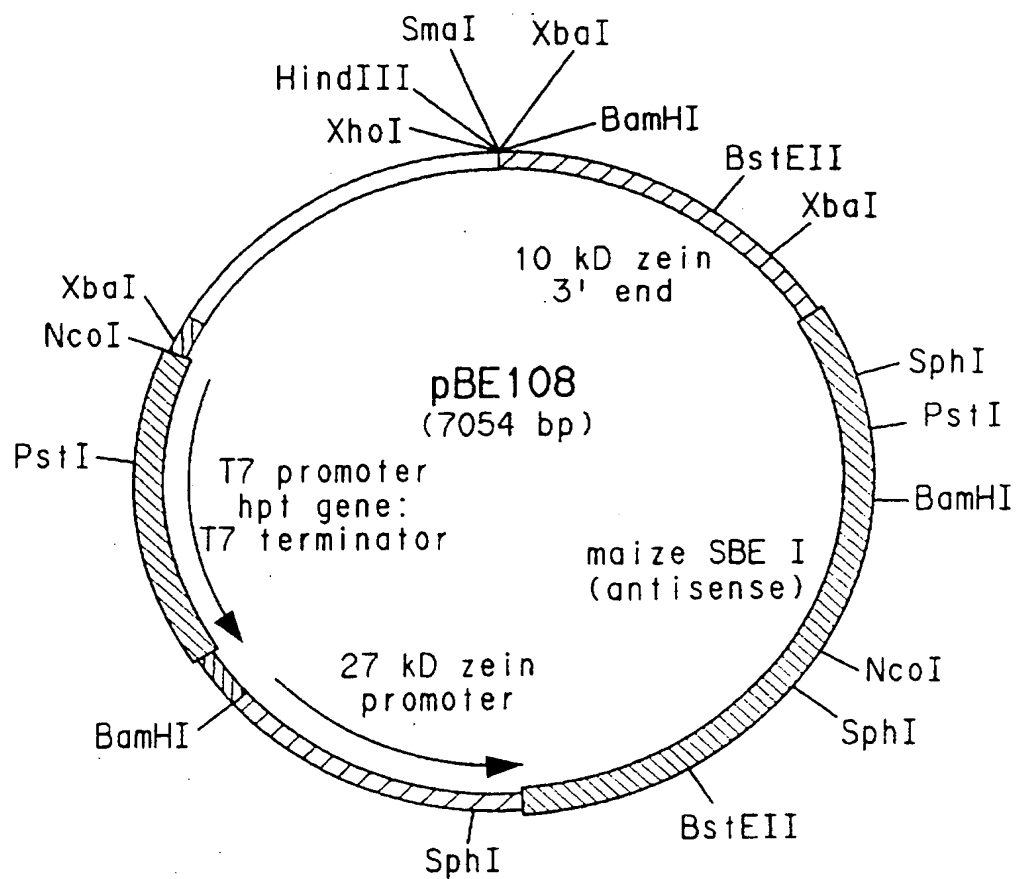


FIG. 12

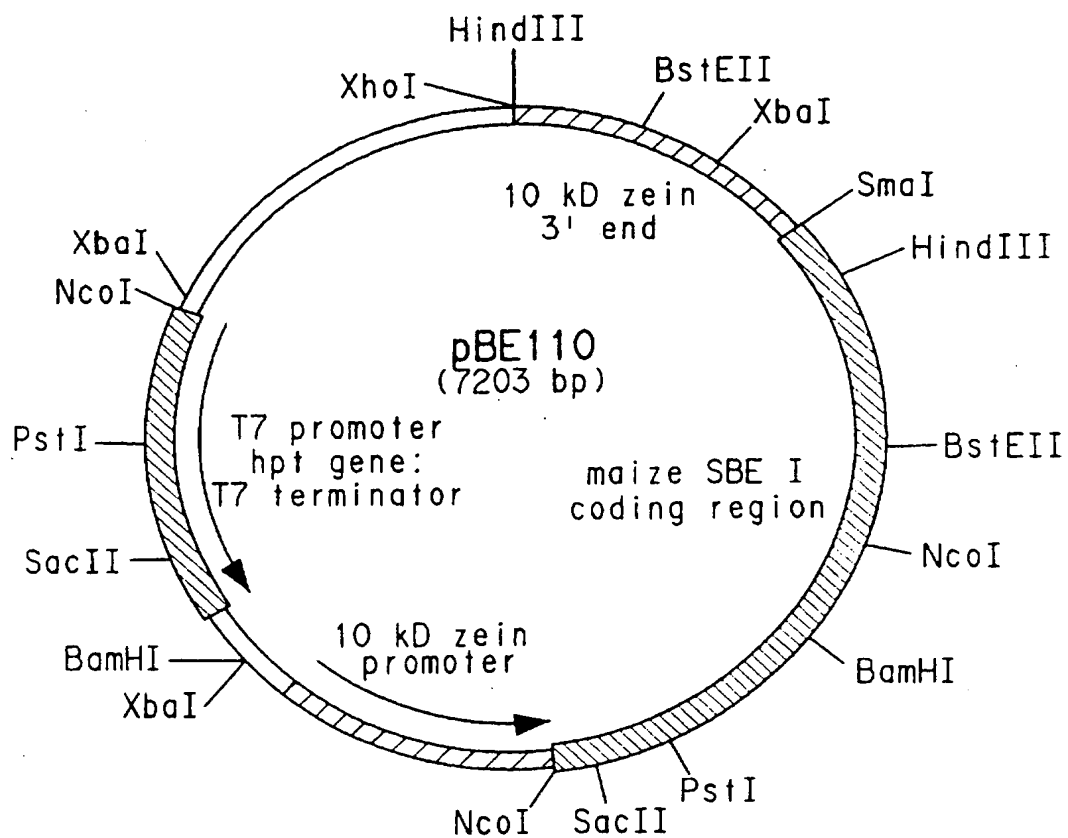
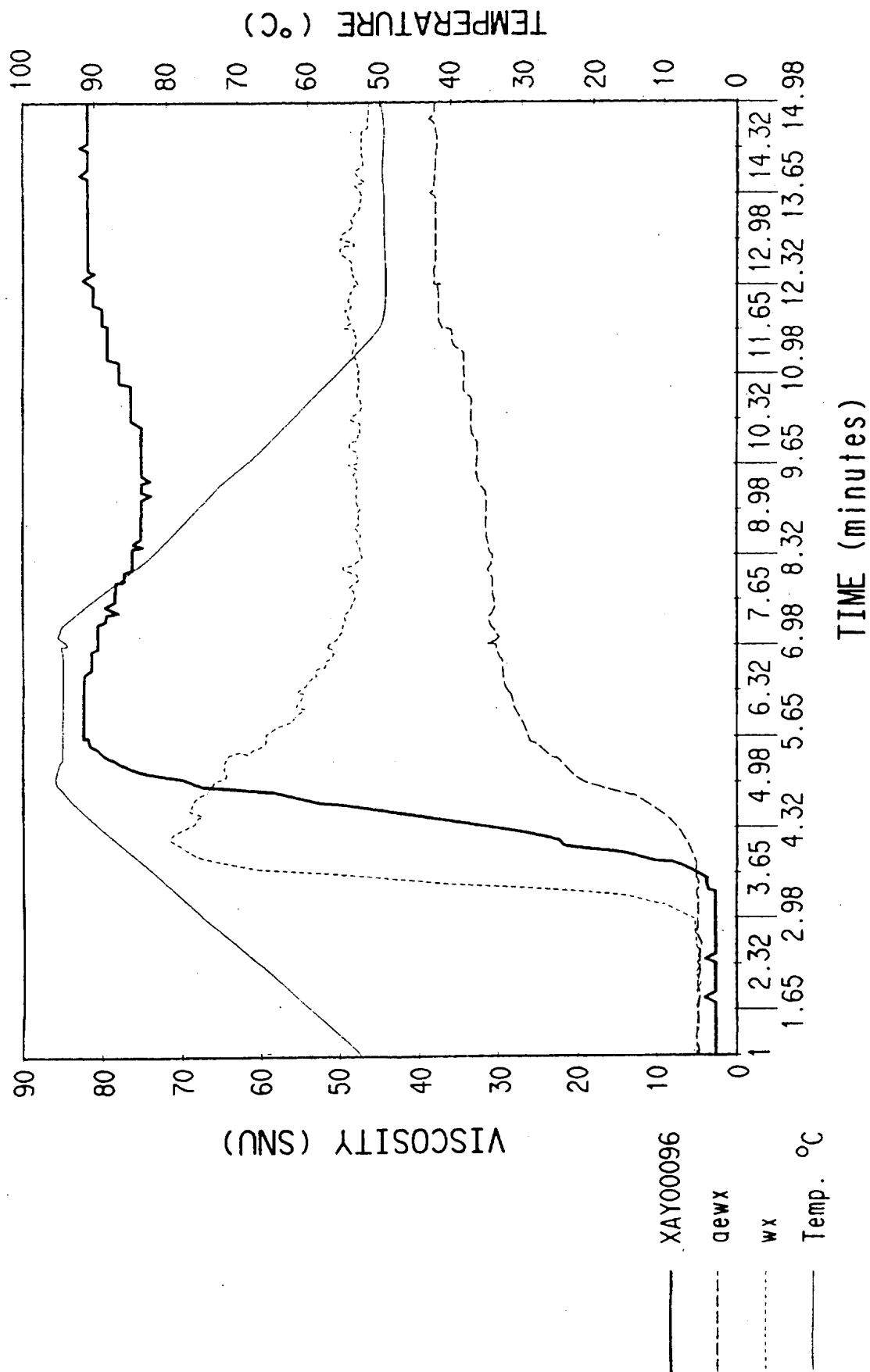


FIG. 14

FIG. 16



Applicant's or agent's file
reference number

1066

International application No.

PCT/US 96/1967

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>22</u> . lines <u>18-20</u>	
B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet <input type="checkbox"/>	
Name of depositary institution AMERICAN TYPE CULTURE COLLECTION	
Address of depositary institution (including postal code and country) 12301 Parklawn Drive Rockville, Maryland 20852 US	
Date of deposit 05 December 1995 (05.12.95)	Accession Number 97365
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/>	
In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC)	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g. "Accession Number of Deposit")	
<div>For receiving Office use only</div> <div><input checked="" type="checkbox"/> This sheet was received with the international application</div> <div>Authorized officer</div>	<div>For International Bureau use only</div> <div><input type="checkbox"/> This sheet was received by the International Bureau on:</div> <div>Authorized officer</div>

Form PCT/RO/134 (July 1992)



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification⁶ : C12N 15/54, 15/82, 9/10, C08B 30/00, A01H 5/00, A23L 1/0522</p>	<p>A3</p>	<p>(11) International Publication Number: WO 97/22703 (43) International Publication Date: 26 June 1997 (26.06.97)</p>
<p>(21) International Application Number: PCT/US96/19678 (22) International Filing Date: 12 December 1996 (12.12.96) (30) Priority Data: 60/009,113 20 December 1995 (20.12.95) US (71) Applicant (for all designated States except US): E.I. DU PONT DE NEMOURS AND COMPANY [US/US]; 1007 Market Street, Wilmington, DE 19898 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): HUBBARD, Natalie, Louise [US/US]; 2204 Mill Lane, Wilmington, DE 19810-4028 (US). KLEIN, Theodore, Mitchell [US/US]; 2229 Rosewood Drive, Wilmington, DE 19810-2815 (US). BROGLIE, Karen, E. [US/US]; 520 Port Royal Court, Landenberg, PA 19350-1333 (US). (74) Agent: MORRISSEY, Bruce, W.; E.I. du Pont de Nemours and Company, Legal Patent Records Center, 1007 Market Street, Wilmington, DE 19898 (US).</p>		<p>(81) Designated States: AL, AM, AU, AZ, BA, BB, BG, BR, BY, CA, CN, CU, CZ, EE, GE, HU, IL, IS, JP, KG, KP, KR, KZ, LC, LK, LR, LT, LV, MD, MG, MK, MN, MX, NO, NZ, PL, RO, RU, SG, SI, SK, TJ, TM, TR, TT, UA, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p>Published With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments. With an indication in relation to a deposited microorganism furnished under Rule 13^{bis} separately from the description. Date of receipt by the International Bureau: 25 February 1997 (25.02.97)</p> <p>(88) Date of publication of the international search report: 24 July 1997 (24.07.97)</p>
<p>(54) Title: NOVEL STARCHES VIA MODIFICATION OF EXPRESSION OF STARCH BIOSYNTHETIC ENZYME GENES</p> <p>(57) Abstract</p> <p>The instant invention discloses utilization of a cDNA clone to construct sense and antisense genes for inhibition of starch branching enzyme enzymatic activity in corn. More specifically, this invention concerns a method of controlling the starch fine structure of starch derived from the grain of corn comprising: (1) preparing a chimeric gene comprising a nucleic acid fragment encoding a starch branching enzyme structural gene or a fragment thereof, operably linked in either sense or anti-sense orientation on the upstream side to a nucleic acid fragment encoding a promoter that directs gene expression in corn endosperm tissue, and operably linked on the downstream side to a nucleic acid fragment encoding a suitable regulatory sequence for transcriptional termination, and (2) transforming corn with said chimeric gene, wherein expression of said chimeric gene results in alteration of the fine structure of starch derived from the grain of said transformed corn compared to the fine structure of starch derived from corn not possessing said chimeric gene.</p> <div data-bbox="695 1123 1461 1764"> </div>		

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/19678

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/54 C12N15/82 C12N9/10 C08B30/00 A01H5/00
A23L1/0522

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C08B A01H A23L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 11520 A (ZENECA LTD ;KEELING PETER LEWIS (GB)) 26 May 1994 see the whole document ---	1,9, 12-14, 18-20
X	WO 94 09144 A (ZENECA LTD) 28 April 1994 cited in the application	1,9,12, 13,18-20
Y	* see the whole document, esp. p.10,1.1-10, p.34,1.2-17, p.53,1.13-35 * --- -/-	2-8,10, 11, 14-17,21



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents :

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- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- *&* document member of the same patent family

Date of the actual completion of the international search

28 May 1997

Date of mailing of the international search report

1 0.06.97

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
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Fax (+ 31-70) 340-3016

Authorized officer

Kania, T

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/19678

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PLANT PHYSIOLOGY, vol. 104, 1994, pages 1449-1453, XP002031588 GUAN H. ET AL.: "Expression of branching enzyme I of maize endosperm in Escherichia coli" * see the whole document, esp. p.1452, last chapter * ---	1-21
A	P.N.A.S., U.S.A., vol. 92, no. 4, 14 February 1995, pages 964-967, XP002031589 GUAN H. ET AL.: "Maize branching enzyme catalyzes synthesis of glycogen-like polysaccharide in glgB-deficient Escherichia coli" see the whole document -----	1-21